Vol. 29, No. 3 September 1952

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The Journal of Experimental Biology

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Published for The Company of Biologists Limited
CAMBRIDGE UNIVERSITY PRESS
CAMBRIDGE, AND BENTLEY HOUSE, LONDON

AGENT FOR U.S.A.
ACADEMIC PRESS INC.,
125, EAST 23RD STREET, NEW YORK, U.S.A.

Price 21s. net *Subscription per volume 63s. net*

THE JOURNAL OF PHYSIOLOGY

SEPTEMBER 1952. VOL. 118, NO. 1

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VOL. 29, NO. 3 SEPTEMBER 1952

SYMPATHOMIMETIC ACTIVITY IN THE ISOLATED FROG'S HEART (*RANA TEMPORARIA*)

BY C. L. SMITH
Department of Zoology, University of Liverpool

(*Received* 28 *August* 1951)

(With Seven Text-figures)

In a previous paper (Smith, 1951) the occurrence of various types of relation between temperature and the pulse rate of the isolated frog's heart was reported. Five main types of curve were observed and their chief features may be briefly summarized as follows. Type *A* showed an exponential relation between frequency and temperature, and corresponded to the 'winter' curve originally described by Barcroft & Izquierdo (1931). Type *B* was rather similar to *A*, but instead of the rates lying along an even curve they lay on two straight lines which intersected at about 10° C. Type *C* showed a linear relation between frequency and temperature over the range investigated and the value of the observed temperature coefficient (Q_{10}) was about 2·5. This form corresponded to the 'summer' curve of Barcroft & Izquierdo (1931). Type *D* was of infrequent occurrence, and in this case the pulse rates for the upper and lower temperatures lay on straight lines of very similar slope, but which had different origins at 7° C. Type *E* again showed a linear relation between pulse rate and temperature, but in this case the observed temperature coefficient was significantly lower than that found for type *C* (2·1). It was further shown that pulse rate curves of type *B* or type *D* could be produced by treatment of type *E* hearts with an extract of anterior pituitary gland in the presence of added adrenaline. Type *C* curves were obtained from type *E* hearts when they were perfused with Ringer containing anterior pituitary extract, adrenaline and thyroxine. To explain these results it was suggested that there was a synergistic action between an anterior pituitary principle and adrenaline which was inhibited at temperatures below 10° C. Further, as types *A*, *B* and *C* pulse rate curves could be obtained from normal hearts in the absence of external adrenaline, the hypothesis was advanced that the isolated heart was under the influence of an active sympathomimetic substance even when it was being perfused with unmodified Ringer.

In the course of further work on this problem experimental evidence has been obtained which throws more light on the genesis of these various pulse rate curves. In the main this work supports the basic hypothesis put forward in the earlier paper, but it is necessary to correct some of the tentative conclusions previously drawn. It is now apparent that the transformation of type *E* to other types of curve is not a specific action of pituitary extract, but can also be obtained by treatment with ascorbic acid or liver extract. In addition, evidence has now been obtained which makes it seem very probable that not only is the isolated heart subject to persistent sympathomimetic activity, but that adrenergic material is synthesized by the heart tissues and the observed form of the temperature-pulse rate curve depends on the level at which equilibrium is established between production and inactivation of this material. In the present paper experiments on the action of certain adrenergic blocking agents on types *A* and *B* hearts are also described. Finally, various forms of temperature-amplitude curves which correspond to the different types of temperature-pulse rate curves are given and their significance discussed.

MATERIAL AND METHODS

The isolation and perfusion of the hearts used in this work was carried out in the manner previously described (Smith, 1951). The hearts were perfused, at a temperature of about 12° C., for at least 1½ hr. before observations were made. Except where otherwise stated, each experiment was started at approximately 7° C., and when a stable rate had been attained, the temperature was progressively raised to the upper limit of the range used (usually 19 or 21° C.). Observations of pulse rate were then made at approximately 2° intervals over the range, the temperature being kept constant at the desired level for 5 min. before a recording was made. A control experiment in which the heart was perfused with either unmodified or adrenaline-containing Ringer was always made initially to determine the form of the natural temperature-pulse rate curve. In the majority of cases the Ringer was then withdrawn from the apparatus and the extract or drug under investigation added. The pH of the medium was checked and readjusted to 7·6, if necessary, by adding phosphate buffer. The modified perfusate was then reintroduced into the apparatus, and the heart perfused for at least 20 min. before recordings of the pulse rate were started. The only case where this procedure was departed from was in those experiments involving the use of ergotoxine. Here 2 l. of Ringer were made up, of which 1 l. was used for the control experiment, while the required amount of ergotoxine ethanesulphonate was added to the other litre. This solution was well shaken at intervals before it was required for perfusion, and as far as could be ascertained the material had completely dissolved.

Liver extract. The extract of frog's liver used in some of the experiments was prepared in the following way. After the heart had been isolated the required amount of liver was weighed out (usually 100 mg.) and ground up with sand. About 100 ml. of alkaline Ringer (pH 8·5) were then added and the material left to extract at room temperature. After the completion of the control experiment this crude extract was added to the perfusate without any further treatment.

Adrenaline. When external adrenaline was required in the perfusate it was added by appropriate dilution of a solution of commercial adrenaline hydrochloride (Liquor Adrenalinae Hydrochloridi, B.P.). This solution contains adrenaline stabilizers in the form of chlorbutol (0·5 %) and sodium metabisulphite (0·05 %).

Mechanical response of the heart. In the majority of experiments kymograph records were taken at each observational temperature, in addition to timing the rate of beat with a stop-watch. The records were obtained by means of an isotonic lever connected to a fine pin in the tip of the ventricle. The movements of the lever were recorded on the drum by a small, glass, frontal writing point. When quantitative data on the mechanical response of the heart were needed for the construction of temperature-amplitude curves the total excursion of the lever was measured. The greater part of this movement was, of course, due to ventricular systole. The same recording system was not used in all experiments, so that no comparison can be made between the absolute heights of the mechanical response of different hearts. In comparing the temperature-amplitude curves for different hearts, attention must therefore be directed to the general form of the curve rather than to the numerical value assigned to the amplitude. The recording conditions remained unchanged throughout a series of experiments on any one heart, and in this case any variations in the absolute heights may be regarded as significant.

EXPERIMENTAL RESULTS

(i) *Modification of type E curves*

In the previous paper (Smith, 1951) the action of extracts of anterior pituitary gland on the linear, low Q_{10}, type of temperature-pulse rate curve was described. It was found that such treatment caused an increased frequency response at higher temperatures so that the temperature coefficient was increased and curves of types *D* or *B* were produced. Numerous experiments have subsequently been made to test the action of ascorbic acid and liver extract on the type *E* heart, and all the results conform very closely with those presented below. In addition, several control experiments have been made to investigate the possibility of spontaneous changes occurring in the form of various pulse rate curves during prolonged perfusion with unmodified Ringer. These have shown that there is no significant change in the relation between frequency and temperature up to 28 hr. after isolation (see also Carter, 1933; Smith, 1951).

Fig. 1 illustrates the typical response of the type *E* heart to liver extract in the presence of external adrenaline. The preliminary control experiment, when the heart was beating in Ringer plus adrenaline (1 in 10^7), showed a linear relation between frequency and temperature over the range 7–17° C. A Ringer-extract of 100 mg. of frog's liver was then added to the medium and the temperature-pulse rate curve determined about 4 hr. later. In this case the frequency acceleration between 7 and 9° C. was very similar to that found initially, but from about 10° C. upwards a marked increase was apparent, so that the final curve consists of two straight lines which intersect at 9·5° C. The value of Q_{10} between 7 and 17° C. increased from 2·21 to 2·60.

Fig. 2 shows the production of a very similar type of curve from an original type *E* by the addition of ascorbic acid (5 mg./l.) to the perfusate. Adrenaline at a concentration of 1 in 2×10^7 was added prior to both experiments. In the case of both anterior pituitary and liver extract the increase in frequency seemed to be confined to the higher temperatures entirely, but there was a definite tendency when ascorbic acid was used for the acceleration of pulse rate between 7 and 9° C. to be slightly higher than in the control experiment. This is the case in the example

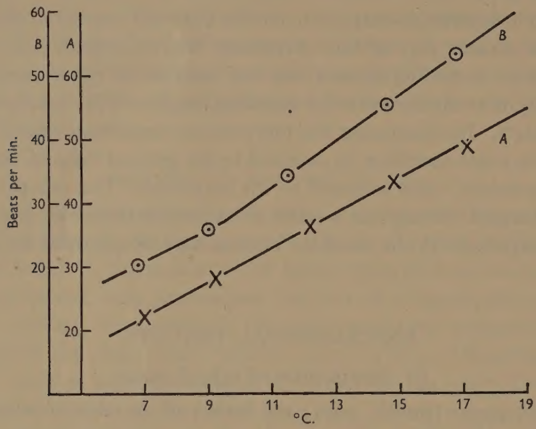

Fig. 1. Action of an extract of frog's liver on type *E* heart, ♂ frog, 9 February 1951. *A*, ×——×, 3½ hr. after isolation. Heart perfused with Ringer containing adrenaline 1 in 10^7. *B*, ⊙——⊙, 7½ hr. after isolation and 2¾ hr. after adding a Ringer-extract of 100 mg. frog's liver to the perfusate. Adrenaline 1 in 10^7.

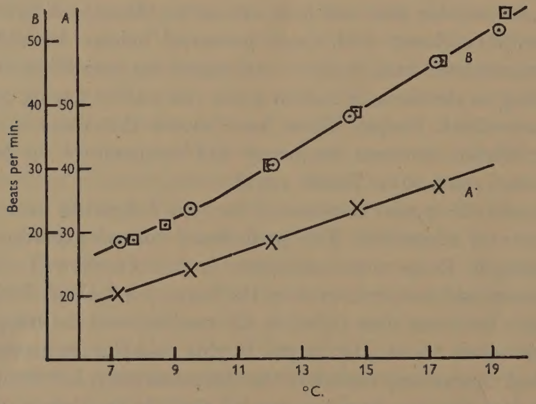

Fig. 2. Action of ascorbic acid on type *E* heart, ♀ frog, 22 February 1951. *A*, ×——×, 3½ hr. after isolation, heart perfused with Ringer containing adrenaline 1 in 2×10^7. *B*, ⊙——⊙, 5½ hr. after isolation and 1 hr. after adding ascorbic acid (5 mg./l.) to the perfusate; adrenaline 1 in 2×10^7. ⊡——⊡, 7½ hr. after isolation, same perfusate containing adrenaline 1 in 2×10^7.

shown in Fig. 2, where the Q_{10} of the initial type *E* line was 1·85, but the projection of the 7 and 9° C. rates to 17° C. in the second experiment would give a line with a Q_{10} of 2·30.

A not uncommon feature shown by hearts in January and February, when the incidence of type *E* curves was high, was the initial rise of frequency on an apparently normal type *E* line, but at about 12° C. the acceleration gradually started to decline and in some cases even became negative, so that the pulse rate at 20° was actually lower than that at 12° C. This effect has been observed with hearts beating in unmodified Ringer as well as in the presence of added adrenaline.

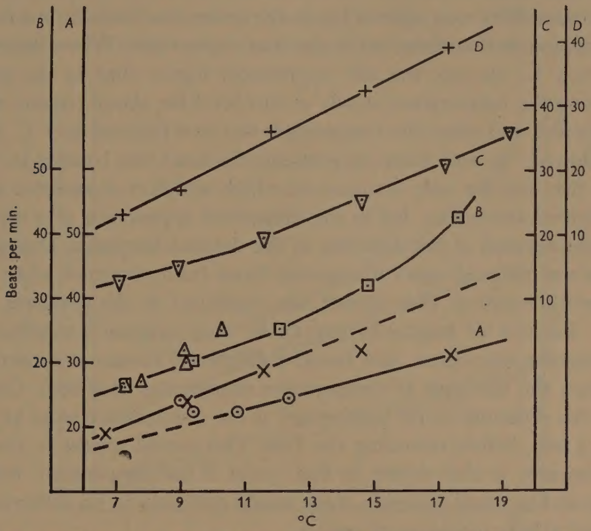

Fig. 3. Action of ascorbic acid on type *E* heart, ♀ frog, 26 February 1951. *A*, 1½ hr. after isolation, heart perfused with unmodified Ringer. ×——×, rates observed when temperature was rising; ⊙——⊙, rates observed when temperature was falling. *B*, 5 hr. after isolation and 2½ hr. after adding ascorbic acid (5 mg./l.) to the perfusate. ⊡——⊡, rates observed when temperature was rising; △——△, rates observed when temperature was falling. *C*, ▽——▽, 24 hr. after isolation, same medium as in *B*. *D*, +——+, 25 hr. after isolation, same medium plus adrenaline 1 in 2×10^7.

Another feature commonly seen in these hearts was the reversibility of this decrease in rate when the temperature was lowered again. A typical curve of this type is shown in Fig. 3, *A*. On raising the temperature from 7° C. the frequency increased linearly up to 12° C., and if it had continued on this course it would have given a Q_{10} of 2·04. There was, however, a marked falling away below this line at the higher temperatures. When the heart was rapidly cooled again the rates at first lay on a lower straight line, but with further temperature reduction there was a relative increase in rate so that the points were coincident with the original line obtained when the temperature was rising. The point on the temperature scale at which this relative increase in frequency appeared was not fixed, but depended on the rate of cooling. For instance, when the heart was quickly cooled to 7° C. the observed frequency was still significantly lower than that observed initially, but if further records were made with the temperature steady at this level, then a progressive increase could be observed until the initial rate was regained.

The action of ascorbic acid on hearts of this type was very revealing, as can be seen from Fig. 3, *B*. After addition of ascorbic acid to the medium, the pulse rate acceleration from 7 to 12° C. again gave a straight line (Q_{10} 2·10), but instead of the frequency falling off at the higher temperatures there was now a marked extra acceleration apparent, so that the curve became type *A*. Furthermore, on quickly cooling the heart there was again a lag in the pulse rate change, but it was in the opposite direction to that observed in the first experiment. When the temperature had fallen to 9° C. the rate was still appreciably higher than in the upward run, but on keeping the temperature steady at this level for about 10 min. the original rate was regained, and when the temperature was now reduced to 7° C. the original path was followed. In both these experiments the heart was beating in unmodified Ringer and this was the only occasion on which addition of ascorbic acid, in the absence of added adrenaline, led to the immediate appearance of a type *A* curve. A very similar reversal of the direction of the delayed frequency change when the temperature was reduced again has several times been observed when the typical action of ascorbic acid or liver extract was produced in the presence of external adrenaline. Barcroft & Izquierdo (1931), in their original experiments on the temperature-pulse rate curve, also found a difference in rate at a particular temperature when the direction of temperature change was reversed. Carter (1933) found that this difficulty could be obviated if the temperature were kept constant for at least 5 min. before recording the rate. This would appear to be due to the same phenomenon as that shown in Fig. 3, for if the temperature were reduced more slowly and by small intervals, there would probably be no difference between the rising and falling temperature curves.

Several experiments have also been made to investigate the action of ascorbic acid on type *E* hearts after more prolonged exposure, both with and without external adrenaline. The record of such a group of experiments is shown in Fig. 4. The control experiment, made 2 hr. after isolation in unmodified Ringer, gave a type *E* curve with some falling off in the rate at higher temperatures which was reversible on cooling again. Another experiment was made after the heart had been beating in the same medium plus ascorbic acid (5 mg./l.) for 2½ hr. The resulting curve (Fig. 4, *B*) is now a quite typical type *E* with no indication of either decreasing acceleration at the higher temperatures or a change-over to type *A* or type *B*. The heart was left under perfusion overnight in the same medium, and another determination made 24 hr. after isolation. It is apparent that there was now a further definite change in the temperature-pulse rate curve (Fig. 4, *C*). The projection of the line joining the 7 and 9° C. rates would give the same coefficient as before (2·16), but there was a marked increase in the rate of beat at the higher temperatures so that the observed coefficient became 2·72. Thus the same result was obtained by longer exposure to ascorbic acid in the absence of external adrenaline as was shown almost immediately when adrenaline was added to the medium (Fig. 2).

Even when a modification of the type *E* curve was produced almost at once by ascorbic acid or liver extract in the presence of adrenaline it was possible to observe a continuing action over a longer period. In the experiments shown in Fig. 5 the control curve (*A*) with unmodified Ringer was a normal type *E* which was unchanged after exposure to an extract of frog's liver for 1½ hr. (curve *B*). The addition of adrenaline (1 in 10^7) to the medium, however, led to the appearance of a type *D* curve (Fig. 5, *C*), this being the same picture as that previously described after treatment with anterior pituitary extract (Smith, 1951). This heart was perfused overnight and a further experiment made 25 hr. after isolation, but without adding any *fresh* adrenaline to the medium. The curve obtained is of the same type as that which was obtainable only in the presence of adrenaline on the previous day (Fig. 5, *D*). Adrenaline (1 in 10^7) was now added to the medium and another determination made. It is apparent that the presence of external adrenaline now carried the development of the pulse rate curve a stage further, as the increase in frequency over the whole temperature range was now linear and the Q_{10} value high (2·38), that is, a type *C* curve was produced. A very similar response to adrenaline after prolonged exposure to ascorbic acid is also shown in Fig. 3. As already mentioned, this heart was exceptional in that a type *A* curve was produced after treatment with ascorbic acid for 2½ hr. in the absence of added adrenaline. The temperature-pulse rate curve given by this heart 24 hr. after isolation and again in the absence of adrenaline is shown at Fig. 3, *C*. This may be regarded as rather more advanced than that obtained the previous day (Fig. 3, *B*), as the increased

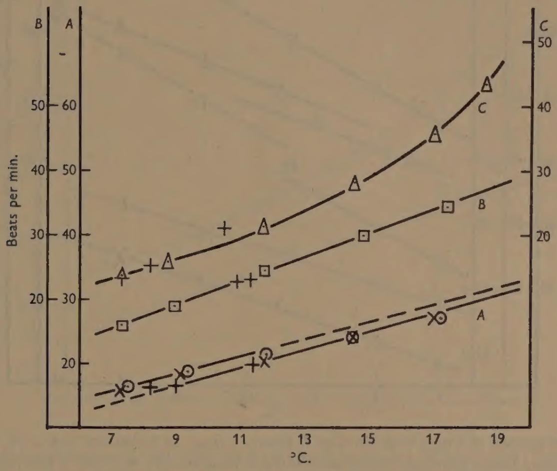

Fig. 4. Action of ascorbic acid on type *E* heart, ♀ frog, 1 March 1951. *A*, ×——×, 2 hr. after isolation; ⊙——⊙, 4 hr. after isolation, heart perfused with unmodified Ringer. *B*, ⊡——⊡, 8 hr. after isolation and 2½ hr. after adding ascorbic acid (5 mg./l.) to the perfusate. *C*, △——△, 24 hr. after isolation, same medium as in *B*. In all three curves rates marked thus, + +, denote a falling temperature gradient.

acceleration appears a little earlier and the general form is type *B* rather than type *A*. On addition of adrenaline (1 in 2×10^7) to the medium, however, the response was very similar to that shown in Fig. 5, as all the observed rates now lie close to a straight line with a Q_{10} of 3·04.

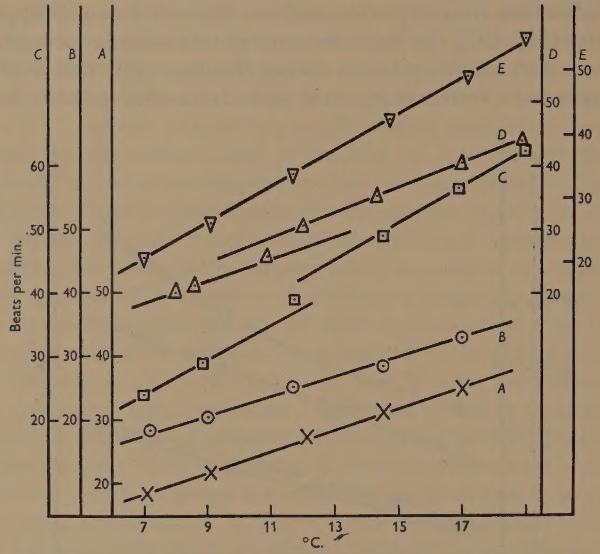

Fig. 5. Action of extract of frog's liver on type *E* heart, ♂ frog, 12 February 1951. *A*, ×——×, 1⅔ hr. after isolation, heart perfused with unmodified Ringer. *B*, ⊙—⊙, 4 hr. after isolation and 1½ hr. after adding extract of 100 mg. frog's liver to the perfusate. *C*, ⊡——⊡, 5½ hr. after isolation, heart perfused with same medium plus adrenaline 1 in 10^7. *D*, △——△, 25 hr. after isolation, perfused with same medium but no fresh adrenaline added. *E*, ▽——▽, 28 hr. after isolation, same perfusate containing adrenaline 1 in 10^7.

(ii) *Action of adrenergic blocking agents*

All the preceding experiments have been directed towards the modification of type *E* curves by treatment with various agents, but it is obviously equally important to ascertain whether the process can be reversed by suitable treatment of types *A* or *C* hearts. In a previous paper (Smith, 1951) preliminary experiments made to investigate the action of an adrenergic blocking agent, ergotamine tartrate, on the temperature-pulse rate curve were mentioned. The results obtained were equivocal, but there was in some cases an indication that the curve was changed in the direction of the type *E* form. Nickerson & Nomaguchi (1950) have subsequently published an account of a detailed investigation into the action of adrenergic blocking agents on the frog's heart. They found that only in the winter could the adrenaline-induced cardio-acceleration be blocked by ergot alkaloids or β-halo-alkylamines. In the summer, however, blockage could be produced if the heart were subjected to the combined action of one of the above agents and sodium fluoro- or iodo-acetate. In view of this the action of ergotoxine, combined with iodoacetic acid, on the temperature-pulse rate curve has been investigated. The record of such an experiment is shown in Fig. 6. This heart was isolated from a frog which had received five injections of sodium thyroxine (0·2 mg. each) during the preceding 10 days. The control curve obtained with the heart beating in Ringer containing ascorbic acid (5 mg./l.) and adrenaline (1 in 10^7) was of the winter or *A* type (Q_{10} 2·60). The frog was given thyroxine and ascorbic acid added to the perfusate to ensure optimal conditions for the appearance of the increased cardio-acceleration at higher temperatures. A perfusate containing ascorbic acid and adrenaline at the same concentration together with ergotoxine ethanesulphonate (1·5 mg./l.) and iodoacetic acid (2·0 mg./l.) was then substituted. The pH of the medium was adjusted to 7·6 immediately before the start of the experiment. It is apparent from Fig. 6, *B*, that the extra acceleration found at the higher temperatures in the first experiment has been largely inhibited, so that the curve is now linear up to 15° C., with a slight rise appearing at 17 and 19° C. (observed Q_{10} 2·09). At the end of the second experiment 0·02 ml. adrenaline (1 : 1000) was added directly to the cannula, and the inotropic and chronotropic response of the heart recorded. The maximum frequency attained is shown in Fig. 6, *B*, and it is evident that there was a definite, though small, response (+2·5 beats per min.). This slight increase in rate after added adrenaline does show, however, that the

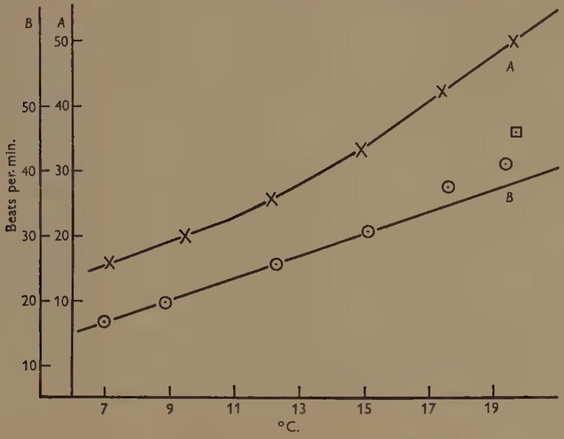

Fig. 6. Action of ergotoxine and iodoacetic acid on the frog's heart, 8 May 1951. Male frog which had received five injections of thyroxine sodium (0·2 mg. each) since 27 April. *A*, ×——×, 3½ hr. after isolation, heart perfused with Ringer containing ascorbic acid (5 mg./l.) and adrenaline (1 in 10^7). *B*, ⊙——⊙, 5 hr. after isolation. Experiment started 30 min. after commencement of perfusion with Ringer containing ergotoxine ethanesulphonate (1·5 mg./l.), iodoacetic acid (2·0 mg./l.), ascorbic acid (5 mg./l.) and adrenaline (1 in 10^7). ⊡, rate observed after adding 0·02 ml. adrenaline (1 : 1000) to the perfusion cannula at the end of the experiment.

chronotropic reaction to adrenaline was not completely blocked, and hence the slight departure from linearity of the second curve in the upper temperature range could have been due to a sympathomimetic action breaking through the blockade. Nickerson (1949) and Nickerson & Nomaguchi (1950) have shown that the ergot alkaloids block the chronotropic response to adrenaline competitively. If, as suggested below, there is increased synthesis of adrenergic material in the heart with rising temperature it is not unnatural to find some adrenergic stimulation breaking through in this way. When adrenaline was added to the cannula at the end of the second experiment (Fig. 6, *B*) there was a very pronounced increase in amplitude. This is in agreement with the work of Nickerson & Nomaguchi (1950) who found that, when the chronotropic response was completely blocked, the inotropic response to adrenaline was quite unimpaired.

Stefan (1928) investigated the action of ergotamine on the isolated frog's heart. He found that while a commercial solution of ergotamine tartrate (1 in 10^4) caused a significant reduction in the rate of beat, a solution of pure ergotamine tartrate of the same strength led to the appearance of a considerably higher pulse rate on warming than was found in any of the control hearts. Stefan stated that difficulty was experienced in dissolving the ergotamine in the second case. In one experiment I also observed a stimulatory action of ergotamine. This heart when perfused with Ringer containing ergotamine tartrate (1 in 10^5) showed little change in rate at the lower temperatures, but above 11° C. there was very marked progressive increase in both pulse rate and amplitude compared with the initial control experiment. It is suggested that these results may be due to exposure of the heart to a stimulatory rather than an inhibitory concentration of ergotamine (Jang, 1941).

(iii) *Temperature-amplitude curves*

The fact that the increased frequency response at higher temperatures shown by experimental and normal hearts was very often accompanied by a noticeable increase in the amplitude of beat (Smith, 1951), led to an examination of a large number of kymograph records to see if there were any consistent differences in the amplitude changes shown by the various types of heart. It was found that, while there was no absolutely fixed form of the temperature-amplitude curve corresponding to each type of temperature-pulse rate curve, there was nevertheless a reasonably good conformation to a typical pattern in each case. Temperature-amplitude curves, which may be regarded as fairly typical, for different pulse rate curves are shown in Fig. 7. Curve *A* is representative of the amplitude changes shown by 'summer' hearts (type *C*), where there is usually a continuous increase over the whole range. There is, however, a distinct indication of an intermediate plateau at about 12° C. which is probably of some significance as it occurred regularly. Curve *B* is based on the record of a 'winter' heart (type *A*), and there is a typical initial *decrease* in amplitude from 7 to 9° C. Above this point there is a sharp increase in amplitude which parallels the extra acceleration in rate found in this part of the range. There is a distinct flattening of the amplitude curve at

17 and 19° C., and in some cases the amplitude recorded at 19° C. was less than that at 17° C. Curve *C* corresponds to a type *E* pulse rate curve which showed no falling off in rate at the higher temperatures. Here again there is a decrease in amplitude over the lower part of the range, and while there is some increase in the mid-region, it is very small compared with that shown by curves *A* and *B*. Curve *D* was obtained from measurements of a record from a heart which showed marked falling off in rate above 12° C. Here the amplitude of the beat falls progressively and fairly steadily over the whole range. It is apparent that this series of temperature-amplitude curves shows a continuous trend towards the reduction of the temperature coefficient of the mechanical response. These curves show the

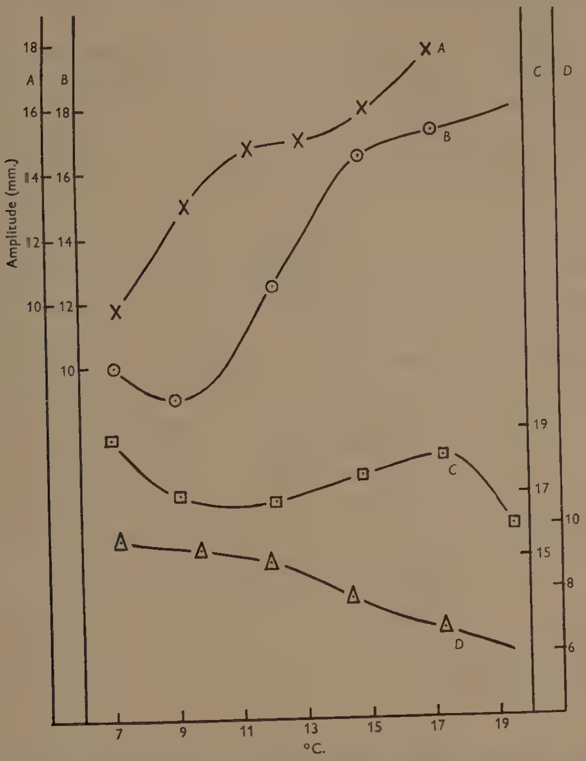

Fig. 7. Temperature-amplitude curves obtained from experiments on isolated frogs' hearts which gave different types of temperature-pulse rate curves. *A*, ×——×, from an experiment which gave a linear, high Q_{10}, pulse rate curve (type *C*); ♀ frog, 18 December 1947. *B*, ⊙——⊙, from an experiment which gave an exponential pulse rate curve (type *A*); ♂ frog, 26 May 1950. *C*, ⊡——⊡, from an experiment which gave a linear, low Q_{10}, pulse rate curve (type *E*); ♂ frog, 11 May 1951. *D*, △——△, from an experiment in which there was marked falling off in pulse rate acceleration at higher temperatures (cf. Fig. 3); ♀ frog, 17 February 1950.

following values for Q_{10} (amplitude) over the range 7–17° C.; curve *A* 1·90, curve *B* 1·73, curve *C* $-1·03$, and curve *D* $-1·40$. It is generally considered that the amplitude of the mechanical response of the heart would be expected to decrease with increasing temperature owing to the added frictional losses at faster rates of contraction (Clark, Eggleton, Eggleton, Gaddie and Stewart, 1938). It would seem, therefore, that curve *D* may be the simplest expression of the relation between amplitude and temperature, although even here there is probably a disproportionate decrease in the mechanical response at higher temperatures. As the reversal of the expected amplitude changes in the other types is also accompanied by an increased frequency response it seems logical to attribute both to the internal release or synthesis of sympathomimetic material.

DISCUSSION

In the previous paper (Smith, 1951) it was suggested that the isolated frog's heart, beating in unmodified Ringer, might be under the influence of sympathomimetic substances of endogenous origin. It is considered that the experimental work described on the preceding pages provides confirmation for this hypothesis. The depression of the extra frequency response in the upper temperature range by a combination of adrenergic blocking agents and the correlation found between the temperature-pulse rate and temperature-amplitude curves strongly suggest that sympathomimetic activity is involved. Perhaps the clearest evidence for the synthesis rather than the release of such material is provided by the anomalous type *E* hearts such as that shown in Fig. 3. In these hearts the frequency at 7° C. appears to be stable and, indeed, on raising the temperature the acceleration at first follows the expected course, but then a progressive and reversible falling away supervenes. Although this falling off is reversible by lowering the temperature again, there is a marked time lag before the original rate is regained. This is consistent with the conception that the original rate at 7° C. is partly due to stimulation of an adrenergic nature, but as the temperature is raised the active substance is progressively inactivated and so the initial acceleration is not maintained. On cooling the heart the rate of inactivation will decrease again, but the adrenergic stimulation cannot attain its former level until resynthesis of active compound has had time to take place. On the basis that in these hearts inactivation or destruction of adrenergic material is the dominant process at higher temperatures, it would be expected that addition of a substance such as ascorbic acid would at least partially protect the active substance. Numerous workers have shown that ascorbic acid has a marked stabilizing action on adrenaline, both *in vitro* and *in vivo* (Bacq, 1936; Welch, 1934; Clark & Raventlos 1939). In the experiment shown in Fig. 3, it might seem that the only process involved was this protective action. Thus, when the temperature is raised in the presence of ascorbic acid, not only is the active material present at the lower temperature stabilized as the temperature rises, but also the higher temperature promotes synthesis of more material, so that the heart shows an increased chronotropic response and an exponential type of curve is produced.

Further evidence that synthesis of active material is promoted at the higher temperatures, in addition to protection from inactivation being afforded, is provided by the behaviour of the heart on rapidly cooling in the presence of ascorbic acid. At about 9° C. the rate was still appreciably higher than that found with a rising temperature. When the temperature was kept constant at this level for about 10 min. the rate gradually fell to the initial value and was then stable. This type of behaviour suggests that sympathomimetic material was present in greater amount at the higher temperatures, and that the excess slowly disappeared after rapid cooling, until the normal balance between production and inactivation for that particular temperature was attained.

The heart shown in Fig. 3 was, however, the only case where an immediate change-over to an exponential curve was produced by ascorbic acid in the absence of external adrenaline. All other hearts examined show more clearly that the action of ascorbic acid is twofold. For instance, it can be seen from Fig. 4 that a type *E* curve which showed slight falling off above 12° C. was first of all converted into a typical type *E* curve by ascorbic acid (Fig. 4, *B*). Not only did this curve not show any decrease in acceleration over the range examined, but also on rapidly cooling again the rate of beat agreed with that observed when the temperature was rising. This is the effect which would be expected if ascorbic acid were simply preventing adrenergic material from being inactivated. After the heart had been beating in the same medium for about 20 hr., however, a further change in the temperature-pulse rate curve was apparent. The acceleration between 7 and 9° C. was unchanged, but with continued rise in temperature the rate began to increase more rapidly, and the curve became exponential or type *A*. When the temperature was again rapidly reduced there was a delayed frequency change similar to that shown in Fig. 3. In cases such as this the delayed production of the exponential curve suggests an action of ascorbic acid or liver extract on the synthesis of endogenous adrenergic compounds. Beyer (1942) found that ascorbic acid promoted the synthesis of sympathomimetic amines *in vitro*, and suggested that it might bring about a step in the synthesis of adrenaline from its precursors. Bourne (1936), after considering the cytological distribution of ascorbic acid, also suggested that it might play a part in adrenaline synthesis.

Previously (Smith, 1951), it was suggested that the action of an extract of anterior pituitary gland on the type *E* temperature-pulse rate curve was due to the occurrence of synergism between an anterior pituitary principle and the exogenous adrenaline which appeared to be necessary for the production of the effect. It is obvious that this hypothesis requires modification in view of the later work. In the first place it is not a specific action of pituitary extract, as very similar effects are obtainable with either ascorbic acid or liver extract. It is not, however, suggested that the action is necessarily due to the same substance in each case. Ascorbic acid is widely distributed in animal tissues, the pituitary gland being one of the richest sources (King, 1938), and it was at first thought that the action of pituitary and liver extracts on the heart might be due to the presence of ascorbic acid. Samples of Antoxylin (Oxo Ltd.), which were proved to be active on type *E* hearts, have been tested for ascorbic acid content by both the dichlorophenol-indophenol and dinitrophenylhydrazine methods (King, 1951). Both methods showed that there was no detectable ascorbic acid in the extract, so that its activity must depend on the presence of some other factor. It has already been pointed out that no change in the temperature coefficient of the basic line joining the rates at 7 and 9° C. was caused by extracts of anterior pituitary gland or liver, even when this coefficient was particularly low. When the control curve showed low values for this coefficient (1·80–2·00) addition of ascorbic acid generally led to its increase to about 2·20. This may perhaps be due to the fact that the protective action is a minor function in the case of the tissue extracts, the main action being on the synthesis of adrenergic material, whereas in the case of ascorbic acid both actions are prominent. In the earlier work (Smith, 1951), when type *E* hearts were treated with Young's pituitary extract in the presence of external adrenaline, the normal response was different from that obtained with ascorbic acid, in that, although an extra frequency response occurred at about 10° C., the pulse acceleration stabilized again on a higher line of low Q_{10} at about 15° C. (cf. Fig. 5 after liver extract). This could also be attributable to the fact that synthesis of adrenergic material was stimulated by the pituitary extract, but as inactivation was not inhibited a new equilibrium was established at a higher frequency level.

The dependence of the immediate appearance of an increased pulse rate at higher temperatures, after treatment with various agents, on the presence of external adrenaline in the medium still requires explanation. It is obvious that the time factor is involved, as external adrenaline is not required when the heart has been treated for longer periods. Usually the required concentration of adrenaline was obtained by adding 0·1 ml. of a commercial adrenaline hydrochloride solution to a litre of Ringer. This operation would also result in the addition of 0·05 mg. of sodium metabisulphite to the medium. The possibility therefore cannot be ignored that it was the stabilizer, rather than the adrenaline itself, which facilitated the more rapid appearance of an increased pulse rate in these experiments. Even if the commercial stabilizer should prove to be ineffective as regards promotion of the increased frequency response it is possible that the added adrenaline would afford some measure of protection to the endogenous material by competing for the inactivating enzymes. The behaviour of the hearts shown in Figs. 3 and 5 after the addition of external adrenaline is not inconsistent with the conception that extra protection of endogenous adrenergic material occurred. The heart shown in Fig. 5 gave a type *D* curve after 3 hr. in a medium containing liver extract and adrenaline. A curve of very similar form was obtained 20 hr. later without adding any fresh adrenaline, but when adrenaline was added at this stage the chronotropic response at higher temperatures was enhanced and a type *C* curve produced. The sequence of events shown in Fig. 3 after treatment with ascorbic acid is rather similar. Here again addition of external adrenaline 22 hr. after adding ascorbic acid caused the transformation of a type *B* into a type *C* curve. It has been shown previously that commercial adrenaline hydrochloride solution alone at this concentration did not change the form of the type *E* curve. It is therefore possible that the above changes, and the more rapid appearance of an increased pulse rate in a type *E* heart, may be due to increased stabilization of the endogenous material synthesized after treatment with ascorbic acid or liver extract. In experiments previously described (Smith, 1951), it was found that such adrenaline solutions could either lead to no change or an increase in the observed temperature coefficient of the isolated heart, and moreover an increase was usually only observed in those hearts where the natural coefficient was relatively low. Carter (1933), using solutions of pure adrenaline, did not find any alteration of the pulse rate curve. Again it seems possible that this discrepancy may be due to increased stability of endogenous material in my experiments. If the presence of a stabilizer in the adrenaline solution used in my original experiments was indeed a factor in the appearance of an increased pulse rate shortly after treatment with pituitary extract, then the use of such solutions was a fortunate chance, as otherwise this action of pituitary extract could easily have remained undetected.

When an analysis of the seasonal distribution of the various types of temperature-pulse rate curves in terms of thyroid activity was made (Smith, 1951), it was assumed that the occurrence of a type *E* curve indicated inactivity of the gland. This conclusion was based on the fact that at that time it had not been possible to obtain type *C* curves by treatment of such hearts with anterior pituitary extracts. It now appears, however, that type *C* curves can be produced, in the presence of external adrenaline, after longer exposure to ascorbic acid or liver extract. In order to clear up this point histological preparations have been made of thyroid glands from a considerable number of frogs which gave type *E* curves. Examination of such sections revealed marked signs of activity in some of the glands. In an inactive thyroid the epithelium is extremely flattened and the follicles large and distended with colloid, whereas in several of these 'type *E*' glands the epithelium was of a columnar type and the follicles were smaller and not turgid with colloid. It is apparent, therefore, that the type *E* curve cannot be regarded as a direct indication of the thyroid state, as the operative conditions for the appearance of this form seem to involve a defect in the synthesis of adrenergic material by the heart tissues, which is apparently not influenced by the thyroid hormone.

An interesting correlation has been found between the thyroid condition and the occurrence of a decrease in pulse rate acceleration at higher temperatures shown by some type *E* hearts. As stated previously (Smith, 1951), type *E* hearts were regularly obtained in January and February. It is now apparent that there is also a fairly high incidence of this form from September to November, but at this time it is only shown by hearts isolated from female frogs. These autumn, female, pulse rate curves are of the linear, low Q_{10} type with no indication of decreasing acceleration in the upper temperature range, and the thyroid glands from these frogs showed marked histological signs of activity. In January and February, however, a considerable proportion of the type *E* curves did show falling off in frequency (Fig. 3, *A*), and all thyroids from such frogs had an obviously more inactive appearance. In those cases where there was no significant departure from linearity in the

January and February type *E* hearts the thyroid had a very similar appearance to that found in the females in the autumn. Further evidence that lack of thyroid hormone is not the primary factor leading to the appearance of a type *E* curve is provided by the failure of addition of thyroxine alone to the medium to change the form of these curves (Smith, 1951). Type *C* curves can be produced from type *E*, in the presence of external adrenaline, either by long exposure to ascorbic acid or by adding thyroxine to the medium in addition to anterior pituitary extract. This suggests that the action of thyroxine is also one which potentiates sympathomimetic activity, so that the type *C* relation is produced earlier in the presence of thyroxine than when the other agents alone are employed. Bacq (1936) has shown that, *in vitro*, thyroxine inhibits the destruction of adrenaline in solution. It seems very probable, therefore, that the action of thyroxine on the pulse rate curve is due to its protective action on sympathomimetic material in the heart. Obviously, if the increased synthesis of such material at higher temperatures is lacking, as it appears to be in the type *E* heart, then addition of thyroxine alone should not alter the form of the curve, except that, in those cases where the rate falls away with increasing temperature, there might be some amelioration of this condition. If the principal role of the thyroid hormone in this connexion is that of a stabilizer of sympathomimetic material in the heart, then the correlation between inability of the type *E* heart to maintain a constant acceleration over the whole temperature range and thyroid inactivity is not unexpected.

ANALYSIS OF THE OBSERVED TYPES OF TEMPERATURE-PULSE RATE CURVE

From the preceding discussion it is apparent that the normally observed relation between pulse rate and temperature is the result of the interaction of several complementary and antagonistic processes. It has been shown that rising temperature affects three more or less independent systems, all of which contribute to the observed frequency at a particular temperature. These three systems may be briefly defined as follows:

(1) Changing temperature has a direct action on the pacemaker mechanism of the heart such that the rate of beat increases with temperature. At the present time there is little direct information available as to the characteristics of this basic relation. It may, however, be tentatively assumed that the temperature-pulse rate curve obtained from hearts isolated from frogs after long-term hypophysectomy is an expression of this basic relation alone (Smith, 1951; and unpublished). These hearts show a linear relation between frequency and temperature over the range 7–21° C., with a mean value for the temperature coefficient (Q_{10}) of about 2·00. The rate of beat at 7° C. is in general fairly constant at about 13 per min.

(2) It has been suggested that much of the experimental work on the heart leads to the conclusion that substances having sympathomimetic properties are synthesized by the heart tissues. Increase in temperature also accelerates this synthesis, so that the resulting chronotropic action is more marked at the upper end of the range. This adrenergic stimulation summates with the natural rate of

the heart so that not only is the mean rate at 7° C. higher (about 17 per min.), but the observed temperature coefficient is also increased. There is some evidence to suggest that changing temperature either acts at two points in the synthesis of active material, or that two independent mechanisms are involved. The temperature-amplitude curves for type *C* or 'summer' hearts usually show a definite plateau at about 12° C. (Fig. 7, *A*), which would seem to indicate that there is an initial potentiation of adrenergic stimulation which is checked at this point, to be followed by a further increase in activity as the temperature rises beyond 13° C. A similar resurgence of adrenergic activity is also shown by many of the temperature-amplitude curves for type *A* and normal type *E* hearts (Fig. 7, *B* and *C*).

(3) The third process, which is antagonistic in its action on the pulse rate to the two preceding, is the inactivation of sympathomimetic material. These reactions are also accelerated by a temperature increase, and in view of the relative constancy of the pulse rate at 7° C. in hearts of all types (Smith, 1951) it would seem that inactivation is depressed to a very low level at such a temperature. The extent to which the observed temperature-pulse rate curve is modified by these inactivation processes is dependent on several factors, which may act either by protecting the active material or by stimulating its synthesis. Anterior pituitary and liver extracts appear to owe most of their activity to their ability to promote synthesis, while thyroxine probably acts by virtue of its 'antioxygéne' properties (Bacq, 1936). Ascorbic acid falls into a different category as it shows a dual action which can be attributed to adrenaline stabilization and promotion of synthesis.

Nickerson & Nomaguchi (1950) put forward a hypothesis to account for the difference in form of the summer and winter temperature-pulse rate curves. This was based on their suggestion that in the winter frog's heart there is only one pathway for the production of the energy substrate which is necessary for the appearance of the chronotropic response to adrenaline. In the summer heart a second labile pathway is also operative. In view of this they suggest that in the winter form the production of utilizable acetate may be the limiting factor for heat-induced cardio-acceleration. The relation between temperature and pulse rate is therefore indirect instead of direct as it is in summer when the supply of energy substrate is not the limiting factor. The experimental results described here and in the earlier paper (Smith, 1951) do not, however, support this theory. There is no indication that energy substrate is the limiting factor at any temperature in the winter heart, as a chronotropic response to added adrenaline was always obtainable and the form of the temperature-pulse rate curve was not necessarily changed. It would also be difficult to account for the occurrence of the type *E* curve on the basis of substrate lack and for its conversion to the other types by the agents employed. Nickerson & Nomaguchi found that this utilizable acetate was a specific energy source for the chronotropic response, and a deficiency did not affect the inotropic response to adrenaline. In this paper, however, it has been shown that the various forms of temperature-pulse rate curve are accompanied by characteristic differences in the temperature-amplitude curve. This would be an unexpected correlation if the form of the temperature-pulse rate curve were dependent on the availability of utilizable acetate. The conception that synthesis or release of sympathomimetic material leads to a chronotropic response which is superimposed on a basic relation between temperature and frequency is in better accord with the experimental results. The existence of the necessary enzyme systems for such synthesis in certain organs is indicated by the work of Beyer, Blaschko, Burn & Langemann (1950), Schmiterlöw (1951) and Blaschko (1942).

A general analysis of the temperature-pulse rate curve based on the available experimental data can be made in the following terms. In all normal isolated hearts the observed rate of beat at 7° C. is partly due to adrenergic stimulation. In the summer heart (type *C*) the active material is protected from inactivation by the presence of thyroid hormone and, in addition, rising temperature has a marked stimulatory action on its synthesis. In consequence, the typical linear relation with a high temperature coefficient is obtained. In the winter heart (type *A*) the level of circulating thyroid hormone is lower, and hence inactivation of the sympathomimetic substance is more rapid. Between 7 and about 12° C. increased destruction under the influence of rising temperature is the dominant process, so that the temperature coefficient over this part of the range is low. At higher temperatures, however, synthesis is increased to such an extent that a positive inotropic and chronotropic response occurs again, and the observed curve is either exponential or in the form of two intersecting straight lines. The curve tends to reach a maximum at about 19° C., which may possibly be due to inactivation again becoming the dominant feature at temperatures higher than this. The fact that this tendency towards a decreasing pulse rate above 19° C. can be obviated by adding thyroxine to the medium (Carter, 1933), also suggests that inactivation of adrenergic material is the underlying cause.

The seasonal type *E* curve, which is linear in form with a low temperature coefficient, would seem to be primarily due to defective synthesis of adrenergic material at higher temperatures. In addition, the effect of inactivation of active material which was present at the lower temperatures can be clearly seen in type *E* hearts which showed a marked falling off in rate above about 12° C. These type *E* hearts may be regarded as providing further indirect evidence for the existence of two independent routes for the synthesis of adrenergic material. It is apparent that synthesis can take place at low temperatures (Fig. 3, *A*), but even when this material is protected by ascorbic acid the increasing chronotropic action at higher temperatures does not necessarily appear at once (Fig. 4, *B*). Although increased synthesis at higher temperatures may not be entirely lacking as judged by the inotropic response (Fig. 7, *C*), it seems that the threshold for the chronotropic response is not attained. As several agents have been found to alter the form of the type *E* curve it is not possible to define the factors leading to the seasonal appearance of this type. Further work on the problem is required to ascertain whether it is due to an endocrine change or a nutritional deficiency.

SUMMARY

1. It was found that addition of ascorbic acid or an extract of frog's liver to the medium perfusing hearts showing a linear, low Q_{10}, temperature-pulse rate curve (type *E*) led to an increased frequency response at the higher temperatures. By such treatment curves of types *A*, *B* or *D* were obtained (Smith, 1951).

2. In nearly all cases it was necessary to add adrenaline (1 in 10^7) to the perfusate to obtain an early response of this nature. In the absence of external adrenaline a similar change was observed after longer treatment with ascorbic acid or liver extract (up to 20 hr.). The possible action of adrenaline in this respect is discussed, and it is suggested that it may afford protection to sympathomimetic substances in the heart tissues.

3. The occurrence of a decreasing acceleration of pulse rate at higher temperatures in certain types of hearts was observed. This phenomenon was reversible on lowering the temperature again, but there was a marked time lag before equilibrium was re-established. When such hearts were treated with ascorbic acid or liver extract, and type *A* or *B* curves produced, the direction of this delayed pulse rate change was reversed. The significance of this behaviour in relation to the hypothesis that sympathomimetic substances are synthesized by the isolated heart is discussed.

4. It is suggested that the observed modifications of the type *E* curve produced by treatment with anterior pituitary extract, liver extract, or ascorbic acid were due to their action in promoting synthesis of adrenergic material by the heart. In the case of ascorbic acid there was evidence for an additional protective action.

5. It was found that treatment of types *A* or *B* hearts with ergotoxine and iodoacetic acid caused a definite change of the temperature-pulse rate curve towards the type *E* form.

6. Temperature-amplitude curves were constructed for numerous hearts of various types, and it was found that distinct forms occurred in correlation with the different types of temperature-pulse rate curves. It has been shown that the frequency and amplitude changes are related in such a way that they can both be attributed to production or inactivation of sympathomimetic substances.

7. The action of thyroxine in modifying the form of the temperature-pulse rate curve is attributed to its protective action on adrenergically active compounds.

8. An analysis of the various forms of temperature-pulse rate curve has been made on the basis of the action of temperature on three independent systems: (i) the pacemaker mechanism of the heart, (ii) the synthesis of sympathomimetic material, and (iii) the rate of inactivation of such material. In the summer heart (type *C*) synthesis is active and the material formed is protected by the relatively high level of circulating thyroid hormone. In the winter (type *A*) form, owing to the lower activity of the thyroid, the effects of inactivation lead to an exponential relation between pulse rate and frequency. Prior to the breeding season and in female frogs in the autumn there is apparently defective synthesis of adrenergic

material and the type *E* relation appears. In January and February this type is often further modified, owing to thyroid inactivity, so that a constant acceleration is not maintained over the whole temperature range.

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OPTICAL CHANGES IN THE MEMBRANES OF THE SEA-URCHIN EGG AT FERTILIZATION, MITOSIS AND CLEAVAGE

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(With Plates 14 and 15)

(*Received* 16 *November* 1951)

INTRODUCTION

Changes in the birefringence of the sea-urchin egg cortex at fertilization, mitosis and cleavage have been described by Monroy & Montalenti. Monroy (1945) describes a decrease in negative birefringence* at fertilization, and also at cleavage, the decrease during cleavage beginning at the poles of the cell. Monroy & Montalenti (1947) describe a cycle of changes, with birefringence falling at fertilization, rising slightly for a time some 20 min. later, rising sharply again in anaphase, and then falling at cleavage.

Changes in the light-scattering properties of the cortex at fertilization have been observed by a number of workers (Runnström, 1928; Moser, 1939; Öhman, 1945). They have been described in detail more recently by Rothschild & Swann (1949), who found, using an ordinary low-power dark-ground system, that an increase in light scattering spread round the surface of the egg from the point of sperm entry, taking about 20 sec.

In the course of other work, we have repeated some of these observations, and made a number of new ones on light scattering, using the eggs of *Psammechinus miliaris* and *Paracentrotus lividus* (there is no obvious difference in the optical properties of the eggs of these two species). We feel that it is of some value to collect these observations together and illustrate them with photographs.

Before describing our results, a word of explanation is needed on our use of the somewhat ambiguous term 'cortex'. Some authors suppose the surface of the sea-urchin egg to be composed of a number of different layers, to which a variety of names are given. We are doubtful of the validity of some of these distinctions (Swann & Mitchison, 1951); at any rate it is certain that diffraction effects at the surface of a large egg make it impossible to distinguish by optical means closely apposed layers of less than 1 or 2 μ thickness. We therefore refer to the surface layer or layers collectively as the cortex, giving the term a rather wider scope than some other writers. In the unfertilized egg there is nothing outside the cortex, as defined by us, except the jelly coat. In the fertilized egg, on the other hand, there is the hyaline layer or hyaloplasm, which is of appreciable thickness. This layer is readily visible, but is obviously extra-cellular; it may be dissolved away by using calcium free sea water and other means, without affecting the ability of the egg to cleave.

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* Throughout this paper we describe the sign of birefringence with respect to the tangent. A negative tangential birefringence is of course the same as a positive radial one.

BIREFRINGENCE CHANGES IN THE CORTEX

The cortex of the unfertilized egg is negatively birefringent with respect to the tangent, and has a radial optical axis (Runnström, Monné & Broman, 1943). The birefringence is normally very weak (Pl. 14, fig. 1), but the cortex also scatters and depolarizes a considerable amount of light, which makes it appear quite bright under a polarizing microscope, particularly at wide apertures. This scattered light is often yellow or orange, and has sometimes been confused with a first-order birefringence colour. It can, however, easily be distinguished from a true birefringence, since it is not affected by compensation.

On fertilization, the weak negative birefringence disappears in the course of a few seconds, leaving the cortex almost completely isotropic (Pl. 14, fig. 2). Within about a minute the fertilization membrane is elevated from the surface (see below).

Some minutes after fertilization the cortical birefringence reappears, negative once again, and by anaphase it reaches a maximum that is slightly greater than in the unfertilized egg. We have not been able to confirm Monroy & Montalenti's (1947) observations of a sharp increase in anaphase. During cleavage, the cortical birefringence decreases first at the poles of the cell, and later at the equator (Pl. 14, figs. 3–8). The birefringence does not disappear completely as at fertilization.

The intrinsic birefringence of the cortex appears to be negative like the total birefringence, but somewhat stronger, so that the form birefringence must be positive. It seems likely, therefore, that the molecular arrangement in the cortex is radial, while the micellar arrangement is tangential. The decrease in total birefringence at fertilization and cleavage could therefore be due to a decrease of order in the radial arrangement of molecules, or an increase of order in the tangential arrangement of micelles. But since eggs imbibed in media of high refractive index show a reduced intrinsic birefringence at fertilization and cleavage, it is likely that there is, in fact, a molecular disorientation. The decrease in intrinsic birefringence at fertilization is shown in Pl. 14, figs. 9 and 10, and at the poles of the cell during cleavage in Pl. 14, figs. 11 and 12.

BIREFRINGENCE OF THE FERTILIZATION MEMBRANE

The fertilization membrane in sea water is positively birefringent and has a radial optical axis (Runnström, 1928). Both form and intrinsic birefringence are positive, since the retardation weakens, but keeps the same sign, on imbibition with high refractive index liquids such as glycerol (Runnström, Monné and Wicklund, 1946), or potassium mercuric iodide. As is well known, the membrane is nearly isotropic when it is first formed and does not show its full birefringence until 10–15 min.

after fertilization (Pl. 14, figs. 13–18). This increase in birefringence is almost certainly related to the increase in toughness of the membrane which also takes place during this period.

BIREFRINGENCE OF THE JELLY COAT

The jelly coat is normally isotropic, but it can be made to show birefringence under the action of a number of substances including various basic dyes (Monné, 1943). We have found that toluidine blue and Janus Green B produce a strong yellowish birefringence at the outer edge of the jelly coat when there is no compensation in the system (Pl. 14, fig. 19). On compensation, a jelly coat stained with toluidine blue goes green and then blue in one pair of quadrants (those in which the slow direction of the compensator points radially), and bright yellow in the other pair. This is due to the anomalous birefringence of the dye, and is found in other tissues where there is an oriented arrangement of dye molecules, e.g. the surface layer of *Amoeba* (Mitchison, 1950). Comparison with the dichroism and the birefringence of toluidine blue crystals shows that the long axis of the dye molecules must be lying tangentially in the jelly coat, but until more is known about the way in which dyes are bound in biological material, it is impossible to say how far this observation agrees with any postulated structure for the jelly coat. The outer layers of the dyed jelly coat show not only the strongest birefringence but also the strongest absorption in ordinary light (Pl. 14, fig. 20). This may indicate that there is a concentration of the jelly material at the outer edge.

BIREFRINGENCE CHANGES IN THE HYALINE LAYER

The birefringence of the hyaline layer is positive with respect to the tangent (Runnström *et al.* 1943). It is weak in the early stages of mitosis, but we have found that it increases sharply during anaphase (Pl. 15, figs. 1–4). It is significant that this is the sequence of events described by Monroy & Montalenti (1947) for the cortex, and since they make no mention of having used calcium-free sea water to remove the hyaline layer, it is possible that in some of their observations they were in fact seeing changes in the hyaline layer. In our experience the birefringence of the hyaline layer, being stronger and of opposite sign, usually obscures the birefringence of the cortex. The birefringence of the hyaline layer does not seem to be much affected at cleavage. The intrinsic birefringence of the hyaline layer appears to be positive.

LIGHT-SCATTERING CHANGES IN THE CORTEX

Light scattered by the cortex varies greatly under different conditions, being largely a question of the angle from which the egg is illuminated. With vertical incident illumination, light comes into the microscope above the objective, and is reflected down on to the egg through the objective by means of a half-silvered cover-slip set at 45° to the axis. Light scattered backwards at about 180° to the incident beam is then picked up by the objective. Polaroids are used to cut out reflexions from the slide and the water surface. Under these conditions the cortex of the unfertilized egg scatters a considerable amount of light, which is sometimes orange in colour (Pl. 15, fig. 5). At fertilization this scattering disappears almost completely (Pl. 15, fig. 6). The cortex then remains dark until the beginning of anaphase, when the scattering reappears quite rapidly (Pl. 15, figs. 7, 8). At cleavage, the scattering disappears once more, though not quite completely. It disappears first at the poles of the cell (Pl. 15, figs. 9–16).

A slightly different form of vertical illumination is given by the Leitz 'Ultropak' system. Here, the light comes in from above, but through a subsidiary annular lens mounted concentrically round the objective. The angle between the incident and scattered light is thus somewhere between 90° and 180°. Under these conditions the cortex of the unfertilized egg appears bright, and becomes dark at fertilization; the scattering also increases at anaphase and falls at cleavage. The changes resemble those visible with true vertical incident illumination, but they are less striking.

When the eggs are illuminated at 90° to the microscope axis there are no obvious changes in the cortex at any time.

Using ordinary dark-ground systems, the angle between the incident and the scattered light is usually about 60°. The cortex of the unfertilized egg then scatters little light, but becomes brighter at fertilization starting from the point of sperm entry (Rothschild & Swann, 1949). During mitosis and cleavage there are no further obvious changes, and the cortex remains bright.

Using patch stops in ordinary condensers it is possible to reduce the angle between the incident and scattered light yet further. Under such conditions both unfertilized and fertilized eggs show a bright cortex, which does not change markedly during mitosis or cleavage.

The significance of these changes in light scattering is uncertain. From what has been said it will be evident that at fertilization there is a decrease in the light scattered at large angles to the incident beam, and an increase in light scattered at small angles. At cleavage there is a similar decrease in the light scattered at large angles, but apparently no corresponding increase in light scattered at small angles. These changes are usually taken to mean an increase in particle size (Oster, 1950), but such a conclusion must be accepted with caution.

The hyaline layer scatters little or no light with any form of dark ground illumination.

CONCLUSIONS

It is difficult to be sure what these birefringence and light-scattering changes mean in molecular terms, but certain conclusions emerge regardless of particular interpretations. There is, first of all, a striking similarity between the changes in the cortex at fertilization and the changes at cleavage. Secondly, the changes at fertilization start at the point of entry of the sperm and later spread right round the egg, while at cleavage the changes start at the poles of the cell and later spread round to the equator. The relevance of these points for an understanding of mitosis, cleavage and fertilization has been discussed briefly by Swann (1952) and Mitchison (1952), and will be dealt with more fully in a later paper.

SUMMARY

1. Changes in birefringence and light scattering of the sea-urchin egg membranes at fertilization, mitosis and cleavage are described and illustrated.

2. There is a close similarity between the changes in the cortex at fertilization and at cleavage.

3. The changes in the cortex at cleavage start at the poles of the cell, and only later spread to the equator.

We should like to express our thanks to the Directors and Staffs of the Marine Stations at Millport, Roscoff and Banyuls for their help in this work.

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EXPLANATION OF PLATES

Plate 14

All photographs are of living eggs except Figs. 9–12.
Psammechinus miliaris = *P.m.*
Paracentrotus lividus = *P.l.*

Fig. 1. Unfertilized egg. *P.m.* Polarized light. Uncompensated.

Fig. 2. Fertilized egg. *P.m.* As Fig. 1.

Figs. 3–8. Cleaving egg. *P.l.* At 3 min. intervals. Polarized light. Uncompensated. Calcium-free sea water. Fertilization membranes removed.

Fig. 9. Unfertilized egg. *P.m.* Fixed in formalin and imbibed in potassium mercuric iodide. Polarized light. Compensated.

Fig. 10. Fertilized egg. *P.m.* As Fig. 9.

Figs. 11, 12. Cleaving egg. *P.l.* As Fig. 9, but uncompensated. Calcium-free sea water. Fertilization membrane removed.

Figs. 13–18. Elevation of the fertilization membrane. *P.l.* Figs. 13–17 at 1 min. intervals after fertilization; Fig. 18 at 15 min. after fertilization. Polarized light. Compensated.

Fig. 19. Unfertilized egg with jelly coat dyed in Janus Green B. *P.l.* Polarized light. Uncompensated.

Fig. 20. As Fig. 19, but ordinary light.

PLATE 15

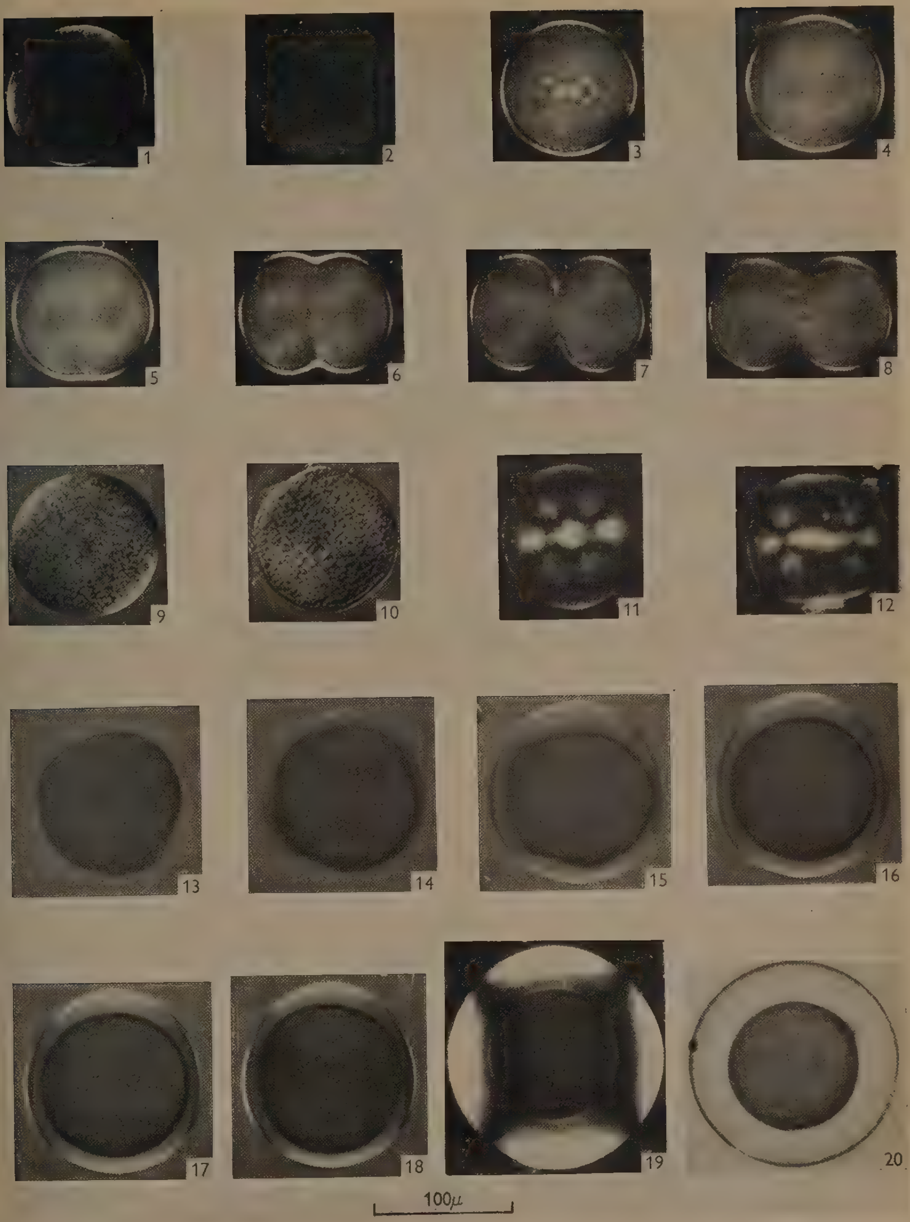
Figs. 1–4. Fertilized eggs, showing the increase in birefringence of hyaline layer during anaphase. *P.l.* At 20, 15, 10 and 5 min. before cleavage. Polarized light. Uncompensated. Fertilization membranes removed.

Fig. 5. Unfertilized egg. *P.l.* Vertical illumination.

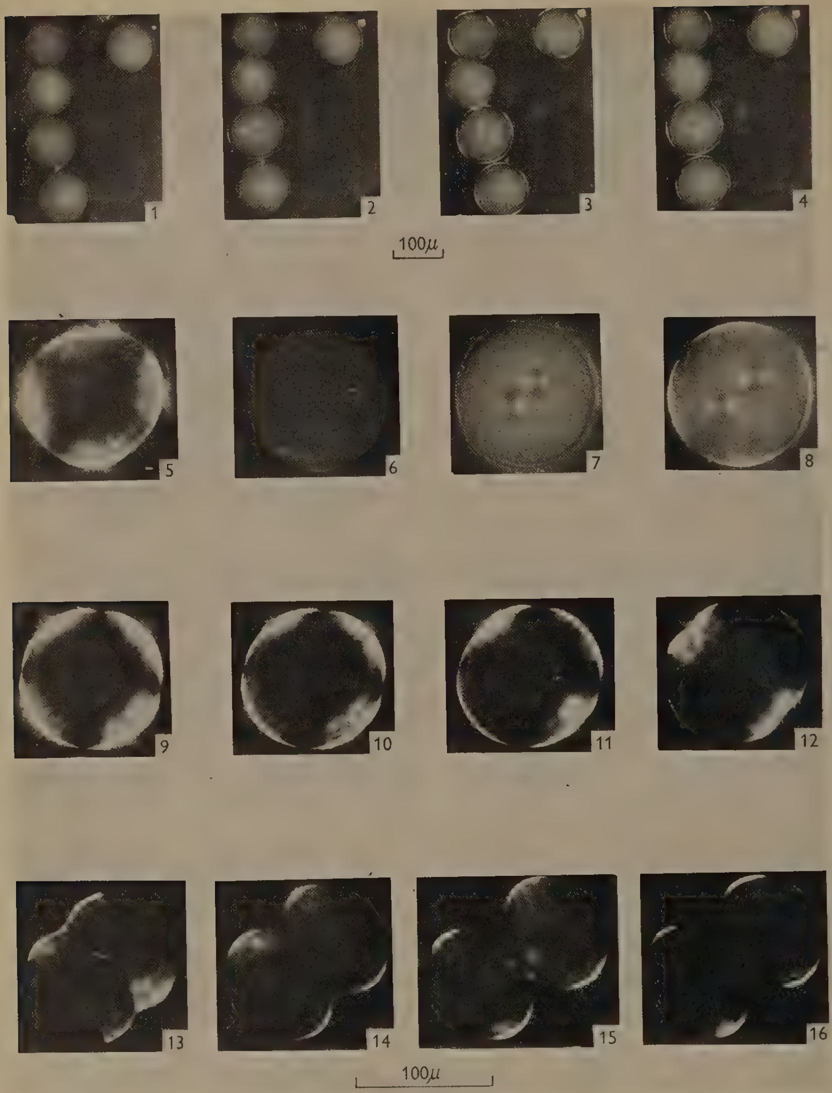
Fig. 6. Fertilized egg. *P.l.* As Fig. 5.

Figs. 7, 8. Fertilized egg, showing the brightening of the cortex during anaphase under vertical illumination. *P.l.* In these photographs, polarized light (compensated) was used together with vertical illumination in order to show the state of the asters. At 12 and 7 min. before cleavage. Calcium-free sea water. Fertilization membranes removed.

Figs. 9–16. Cleaving egg. *P.m.* At 1 min. intervals. Vertical illumination.



MITCHISON AND SWANN—OPTICAL CHANGES IN THE MEMBRANES OF THE SEA-URCHIN EGG



MITCHISON AND SWANN—OPTICAL CHANGES IN THE MEMBRANES OF THE SEA-URCHIN EGG

THE PHYSIOLOGY OF CONTRACTILE VACUOLES

VIII. THE WATER RELATIONS OF THE SUCTORIAN *PODOPHRYA* DURING FEEDING

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(*Received* 5 *November* 1951)

(With Four Text-figures)

INTRODUCTION

It has long been suspected that in certain predatory Protozoa the extra activity of the contractile vacuole during feeding may serve to remove water taken in as part of the protoplasm of the prey (Kitching, 1938). The frequency of systole of the contractile vacuole of the suctorian *Dendrocometes paradoxus* was found by Pestel (1931) to increase greatly during feeding. More recently, Rudzinska & Chambers (1951) have found that the rate of vacuolar output of *Tokophrya infusionum* also increases greatly during feeding. These authors have attempted to draw up a balance sheet of the exchanges of water. They have shown that the decrease in volume of the prey is greater than the increase in volume of the predator, but their balance sheet is vitiated by the fact that in any one experiment only five readings were taken of the rate of vacuolar output during feeding. In the case of *Podophrya* sp. so small a number of readings would be useless, because the rate of output often changes considerably during the course of a meal. Therefore in this paper an attempt is made to present all the information needed for a balance sheet. In addition, experiments have been carried out on the effect of solutions of sucrose on the body volume and rate of vacuolar output of feeding and fed *Podophrya*, for comparison with results previously obtained on unfed individuals. It was hoped that in this way any substantial change in the internal osmotic pressure, which might be brought about by feeding, would be detected; such a change would of course affect the allowance to be made in the balance sheet for osmotic inflow.

The body surface of the suctorian *Podophrya* expands actively during feeding (Kitching, 1952), thereby no doubt lowering the hydrostatic pressure of the cytoplasm, and possibly even creating a suction. The results reported in this paper make it possible to assess also the part played by the contractile vacuole in baling out water derived from the prey, thus making room for still more food.

METHODS

Podophrya sp. was grown on silk threads by the method already described (Kitching, 1951). Material was used from cultures 5–12 days old. In some experiments tap water was used as the medium, and irrigation was automatic (Kitching, 1934). In other experiments the medium was taken from the *Podophrya* culture itself and irrigation was carried out by hand pipette every few minutes. The experiments were carried out at room temperature, which lay between 15 and 20° C., and did not fluctuate by more than ±0·5° C. in any one experiment.

The methods of observation of the contractile vacuole and of computation of the results were the same as those used previously (Kitching, 1934). The body volume of the *Podophrya* was calculated from the major and minor axes as that of a prolate spheroid, and that of the prey was estimated from plasticine models.

In the initial observations *Paramecium caudatum* was used for prey. However, in these cases, towards the end of the meal the *Podophrya* became so filled with granules that it was no longer possible to see the contractile vacuole. For this reason in most experiments the size of the meal was limited by the use of the much smaller ciliate *Colpidium* sp. In either case, when the *Podophrya* was to be fed, a drop of the culture containing the ciliates was placed at the edge of the cover-slip. As soon as the *Podophrya* under observation had caught a ciliate the preparation was re-irrigated with the proper medium (culture fluid or tap water). Normally prey was captured within 1 or 2 min.

RESULTS

Effects of feeding on vacuolar output

In all of the many experiments carried out there was a several-fold increase in the rate of vacuolar output of *Podophrya* within a few minutes of capture of prey. Typical examples are illustrated in Figs. 1 and 2. The course of the increase varied from one experiment to another, as will be seen from these two figures; the peak output was sometimes reached quickly, sometimes slowly. In those cases in which the body surface of the *Podophrya* became wrinkled after the capture of prey, as already described (Kitching, 1952), the rate of vacuolar output increased considerably before the wrinkles had become completely smoothed out owing to the uptake of food. In all cases the vacuolar output declined towards the end of the meal, and on release of the prey became fairly steady at a level still considerably above where it had stood before the meal.

Irrigation of *Podophrya* with *Colpidium* culture fluid from which the *Colpidium* had been separated by centrifuging produced no significant effect on the rate of vacuolar output; nor did the vacuolar output change in normal experiments in which a *Colpidium* was momentarily caught but broke loose.

Balance sheets for exchanges of water during feeding

For the experiments considered in this section *Colpidium* sp. was used as food, and liquid from the *Podophrya* cultures as medium. Within a few minutes of capture material began to flow from the *Colpidium* through the tentacles of the *Podophrya*. From this time the *Podophrya* increased in size steadily, and the *Colpidium* decreased. By the end of the meal the *Colpidium* was reduced to a spherical remnant. At this stage the contractile vacuole of the *Colpidium* stopped working, and there was no further sign of life. The remnant then fell away from the tentacles of the *Podophrya*, although sometimes it swelled a little before doing so. From this time the volume of the *Podophrya* remained unchanged within the limits of measurement for the duration of the experiment; this is illustrated in Fig. 1.

The balance sheets given in Table 1 cover the period between the capture of prey and attainment by the prey of its smallest size, at or slightly before its release. When several *Colpidium* were captured by a *Podophrya* the period used for the balance sheet was determined by the first capture and the last release. The six experiments summarized in Table 1 are the only ones for which it is possible to make reliable estimates of all the items of the balance sheet. In other cases the *Podophrya* or prey did not remain within a plane of focus, and so could not be measured.

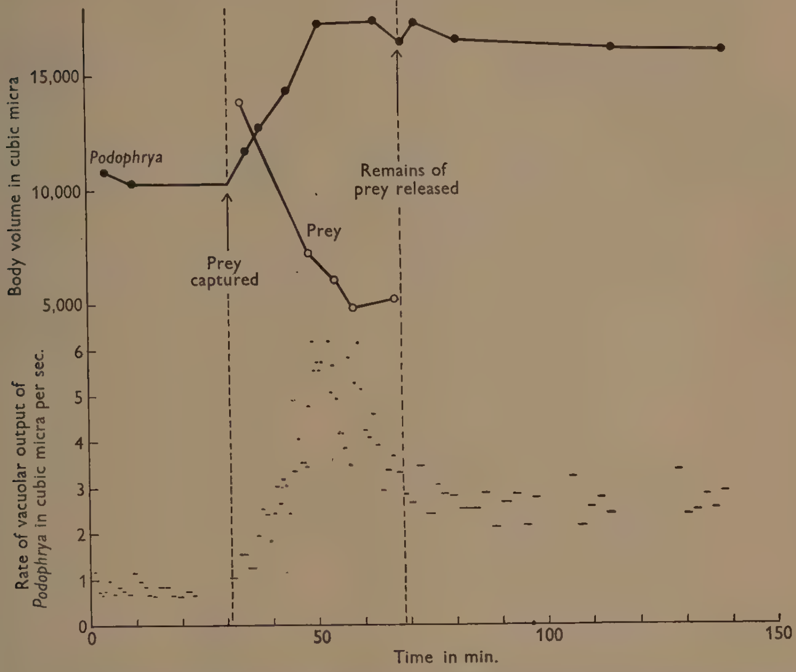

Fig. 1. Changes in the body volume of *Podophrya* and of its prey (*Colpidium* sp.), and in the rate of vacuolar output of *Podophrya*, during a meal of small extent. The water exchange for this experiment is summarized in the last column of Table 1.

The balance sheets enable a comparison to be made between the decrease in volume of the prey on the one hand and the increase in volume of the *Podophrya* plus the extra output of the contractile vacuole during feeding on the other. The extra vacuolar output was estimated from the area enclosed by the curve relating rate of vacuolar output to time, with an appropriate deduction for the output which would have occurred had no prey been given. The curve in question was drawn through points calculated from averages of the maximum diameter and of the intervals between systoles for groups of (usually) six vacuolar cycles. A further correction has also been made for a steady rise of the base-line from the level of vacuolar output before feeding to the level found at the end of the meal. This correction reduces the estimate of the extra vacuolar output somewhat, but is relatively small, and merely serves to show that considerations of this sort are not important. The experimental error is, of course, considerable, but the extra vacuolar output accounts fairly well for the amount by which the swelling of the *Podophrya* falls short of the shrinkage of the *Colpidium*.

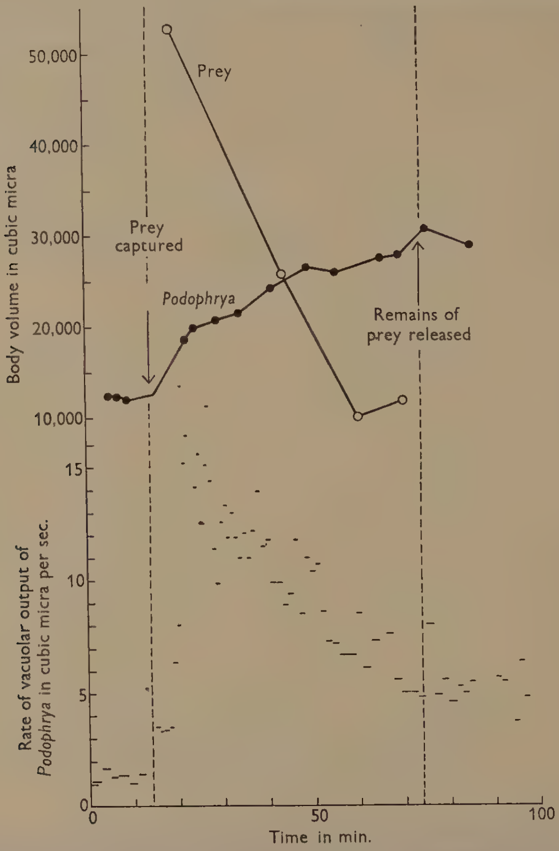

Fig. 2. Changes in the body volume of *Podophrya* and of its prey (*Colpidium* sp.), and in the rate of vacuolar output of *Podophrya*, during a moderate meal. This experiment is the third in Table 1.

The part played by the contractile vacuole in exchanges of water during feeding appears to have been least important when the meal was a small one, as in the

Table 1. *Balance sheet for water relations of* Podophrya *during feeding*
(Volumes are given in cubic micra.)

of prey (*Colpidium* sp.) ure	44,000 38,000 48,000	130,000	68,000 34,000 45,000	147,000	53,000	35,000 35,000 49,000 39,000	158,000	20,000	14,000
of remnants of prey d	33,000 25,000 29,000	87,000	27,600 17,700 23,700	69,000	12,000	16,000 16,000 17,000 14,000	63,000	10,000	5,000
e of volume of prey meal	**43,000**		**78,000**		**41,000**	**95,000**		**10,000**	**9,000**
of *Podophrya* at start of	22,600		26,000		12,200	60,000		20,000	10,600
of *Podophrya* at end of	49,400		76,000		29,600	120,000		28,000	16,500
of volume of *Podophrya*	27,000		50,000		17,000	60,000		8,000	6,000
output of contractile of *Podophrya* during	19,000		42,000		27,000	51,000		6,000	7,000
e correction	−6,000		−11,000		−7,000	−16,000		−2,000	−2,000
d extra output of con- vacuole of *Podophrya* feeding	13,000		31,000		20,000	35,000		4,000	5,000
increase + corrected acuolar output of *rya*	**40,000**		**81,000**		**37,000**	**95,000**		**12,000**	**11,000**

Table 2. *Summary of three experiments in which* Podophrya *was irrigated with solutions of sucrose after a meal*

entration of e in medium (M)	Average rate of vacuolar output (μ^3/sec.)	No. of readings on which average is based	Duration of treatment (min.)	Body volume (μ^3)
efore feeding)	1·4	7	7	17,600
uring feeding)	Max. 5·6	6*	48	—
fter feeding)	2·2	12	24	27,300
	Decreased to zero	—	49	No detectable change
	1·9	15	23	—
efore feeding)	1·2	6	10	19,300
uring feeding)	Max. 4·5	6*	65	—
fter feeding)	1·8	8	34	28,000
	Decreased to 0·05	—	38	Very slight shrinkage
	1·5	24	60	Restored
	0·1	4	55	No detectable change
	1·9	18	100	
efore feeding)	1·8	7	9	21,000
uring feeding)	5·1	6*	48	—
fter feeding)	3·4	33	65	26,000
	Declined from about 1·5 to 1·1	8 (for final average)	65	No detectable change
	3·3	20	65	
	0·0	—	56	Very slight shrinkage
	2·3	19	63	Restored

omplete or almost complete record was kept throughout the meal. The maximum rate of output during feeding was
ed by averaging measurements for the six consecutive systoles with the highest rate of output.

experiment illustrated in Fig. 1 (and given last in Table 1). It will be noted, however, that even in this case towards the end of the meal the *Podophrya* was no longer increasing in size although the prey was still decreasing; the contractile vacuole was then at its peak of activity. A more typical experiment is shown in Fig. 2 (given third in Table 1), in which the rate of decrease of the prey considerably exceeded the rate of increase of the *Podophrya* throughout the meal.

Osmotic relations during and after feeding

In twelve experiments *Podophrya* was transferred, during or after feeding, to a solution of sucrose in tap water. The concentration of sucrose ranged from 0·025 to 0·1 M, and in some experiments several concentrations were used. Results were variable, but in all cases there was a considerable fall in vacuolar output.

Results for three experiments on feeding *Podophrya* are shown in Figs. 3 and 4. A 0·05 M solution of sucrose in one case reduced the vacuolar output almost to zero (Fig. 3*b*), but in another only by about 40% (Fig. 3*a*). In the former case the uptake of food through the tentacles ceased during the treatment with sucrose, but in the latter it continued actively.

In observations on *Podophrya* transferred to 0·05 or 0·075 M-sucrose shortly after a meal, it was found that in some cases the contractile vacuole continued operating, though at a reduced rate of output, and there was no detectable shrinkage of the body. An example is shown in Fig. 4. In other cases the contractile vacuole almost stopped working, and the body shrank slightly, in 0·075 M but not in 0·05 M-sucrose. Examples are summarized in Table 2.

DISCUSSION

Water balance

The normal internal osmotic pressure of an unfed *Podophrya* under the experimental conditions of this work is that of a 0·04–0·05 M solution of sucrose (Kitching, 1951). The rate of vacuolar output increases several-fold during feeding. This increase might be due to a corresponding increase in the osmotic difference between the organism and its medium. However, this is disproved by the fact that a solution of only 0·05–0·075 M concentration can greatly reduce the vacuolar output during feeding. If the internal osmotic pressure of the organism had been increased several-fold by feeding, the effect of sucrose in these concentrations would have been slight. In fact the results illustrated in Fig. 3 suggest that a 0·05 M solution of sucrose is almost sufficient to stop osmotic inflow, and that in these circumstances vacuolar activity depends almost entirely on the uptake of food.

However, the results obtained on fed *Podophrya*, shortly after release of the remains of the prey, suggest that there is an increase in the internal osmotic pressure during feeding from an initial level of 0·04–0·05 M-sucrose (Kitching, 1951) to a final level ranging from 0·05 to 0·075 M-sucrose. This and the increase in surface area of the body would largely account for the higher rate of vacuolar output after

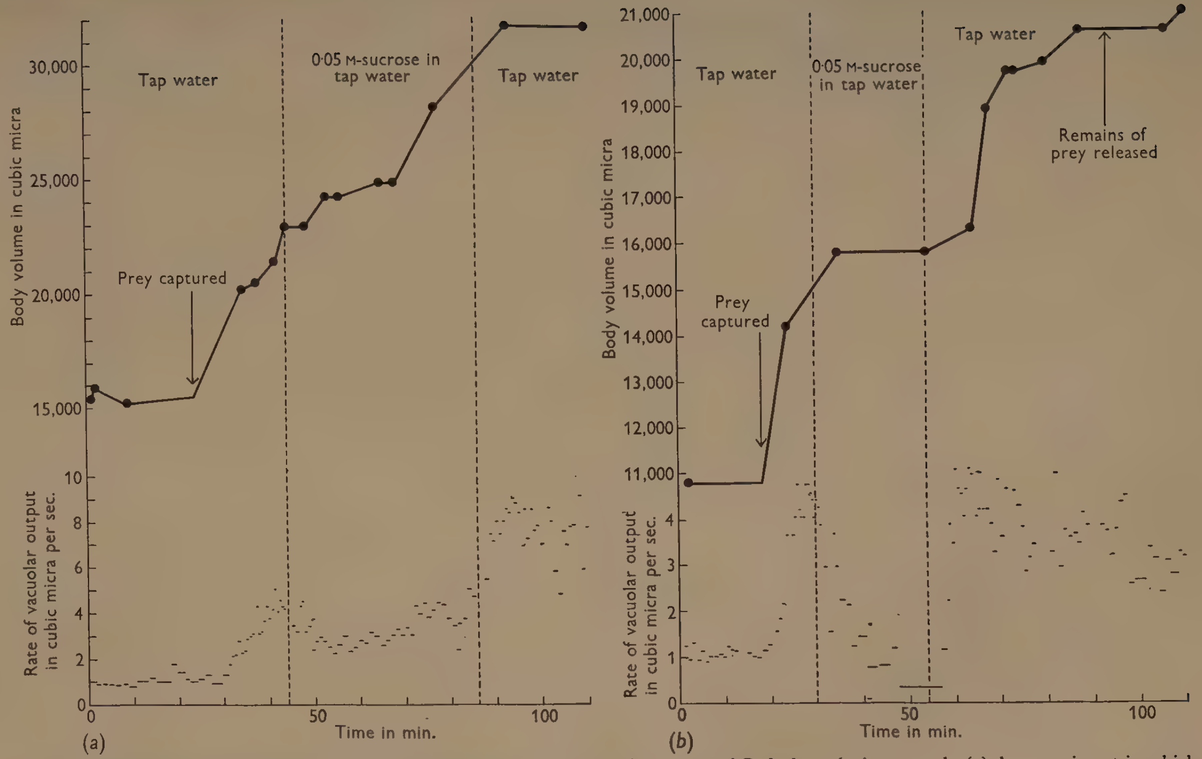

Fig. 3. The effect of 0·05 M-sucrose on the body volume and rate of vacuolar output of *Podophrya* during a meal. (*a*) An experiment in which the uptake of food material through the tentacles was seen to continue actively during the treatment with sucrose. (*b*) An experiment in which uptake of food ceased during the treatment with sucrose.

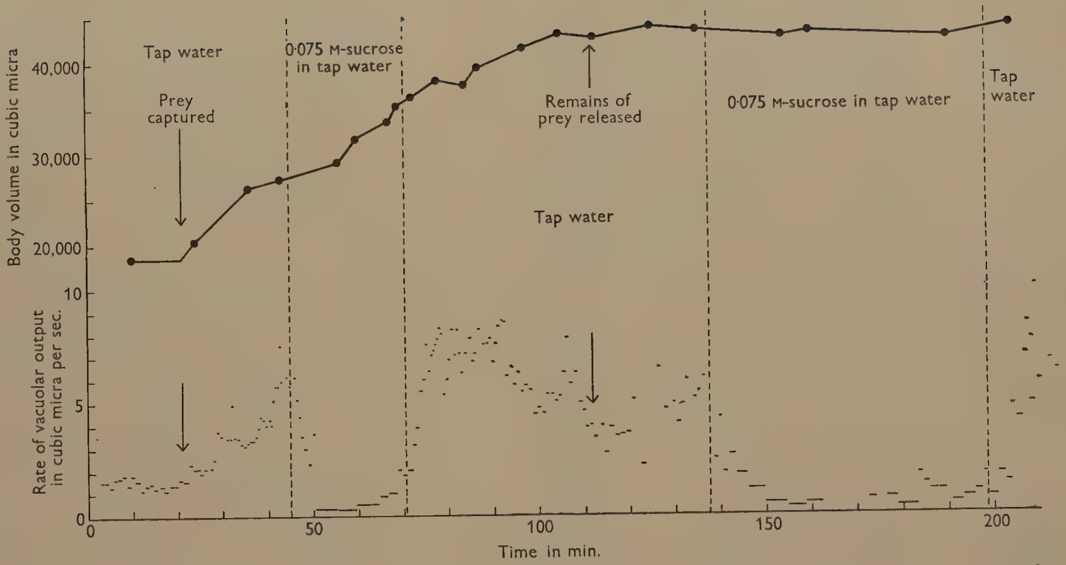

Fig. 4. The effect of 0·075 M-sucrose on the body volume and rate of vacuolar output of *Podophrya* during and after a meal.

feeding as compared with before feeding. There might possibly also be a change in permeability. In the balance sheets of water exchange during feeding (Table 1) the effects of all these changes are taken into account in the base-line correction.

The process of feeding

Capture of prey produces in *Podophrya* an expansion of the body surface (Kitching, 1952), which allows a volume increase of 100% or even more, as will be seen from Table 1 of the present paper. Uptake of food stimulates an increase in vacuolar output, which, in harmony with previous conclusions as to the control of vacuolar output (Kitching, 1951), may be ascribed to the passage of water from the ingested food into the cytoplasm, with consequent increased hydration of the latter. This increase of vacuolar output makes room within the body of the suctorian for a still further quantity of food, also very roughly equal in volume to the original *Podophrya*. Thus the *Podophrya* may ingest about twice its own volume of food, roughly half by expansion and half by concentration of the food material taken in. In any case the contractile vacuole clearly plays an important part in helping to maintain the reduction of pressure produced by the expansion of the body surface. It is not known whether or not it also eliminates dissolved waste matter resulting from feeding or digestion.

SUMMARY

1. The rate of output of the contractile vacuole of the suctorian *Podophrya* sp. was found to increase during feeding up to ten times. It decreased at the end of the meal but remained higher than it was before feeding.

2. The extra output of the contractile vacuole during feeding was approximately equal to the decrease in volume of the prey minus the increase in volume of the *Podophrya*.

3. In a typical meal roughly one-half of the food ingested by *Podophrya* was accommodated by expansion, and one half by concentration of the food taken in. This concentration was brought about by the action of the contractile vacuole.

I am grateful to Prof. J. E. Harris for suggestions and criticisms, and to Mr Edward Livingstone for a steady supply of cultures.

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THE RESPONSES OF THE HONEY-BEE TO REPELLENT CHEMICALS

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(*Received* 25 *July* 1951)

(With Four Text-figures)

INTRODUCTION

This paper is concerned with the development of techniques for the study of the chemotropic responses of the honey-bee and their application to the study of the effect of adding a repellent to a solution of an attractant.

DEVELOPMENT OF THE BASIC TECHNIQUE

The use of olfactometers such as those used by Marshall (1935) for the study of olfactory responses in the honey-bee has been criticized in that such methods can only measure responses under conditions when active flight and defaecation are inhibited. Further, one does not know how far the responses of such a highly developed social insect are modified by the presence of other bees.

Whilst experiments conducted in the field enable observations to be made on naturally flying bees, they suffer from all the inherent disadvantages of involving a large number of factors which are not under control, and the conditions are therefore difficult to reproduce.

Von Frisch (1934) investigated the extent to which external factors influence the threshold responses of the bee to various sugar solutions. He found that with foraging bees over the range of 10–30° C. temperature changes had little effect, but that variations in the availability of naturally secreted nectar of a sufficiently high concentration materially affected the threshold response; during seasons when there was a good nectar flow the bees would refuse a sugar solution which they would readily accept under less favourable conditions even though, under the latter conditions, there was plenty of food in the hive and the bees were equally well fed.

Any evaluation of the rejection thresholds of unacceptable compounds would naturally depend on the comparison of these thresholds with a standard and constant response and therefore would not be possible where the test bees had access to fluctuating sources of nectar.

Another factor which is difficult to control in such field experiments is the number of bees. The sudden cessation of a nectar flow often results in the swamping of the training table by thousands of bees and accurate observations are rendered impossible.

Beling (1929) installed a small colony in a room for purposes of investigating the time sense of the bees, and this idea of restricting the foraging area of the bees was considered in an attempt to combine the advantages of the olfactometer and field techniques with a minimum of the disadvantages inherent in both.

Preliminary experiments indicated that a greenhouse might serve this purpose, and a small house approximately 12 × 8 ft. was adapted by screening the ventilation lights with muslin and installing circulating fans and heaters which provided temperature control within 5° C.

A small nucleus box containing two frames of bees, brood, and a 2-year-old queen was introduced into the greenhouse, and after a few hours of incessant flying the colony settled down. Dishes of syrup placed within the house were discovered by the foraging, bees, and except that large numbers of bees crowded on the glass of the roof during the hotter parts of the day no unusual activity was observed in the colony. This colony of bees installed in the greenhouse was used in the following experiments and, except for the introduction of a fresh comb containing sealed brood and pollen every fortnight, it was not disturbed.

I. THE EVALUATION OF THE RESPONSE WHEN THE REPELLENT IS INCORPORATED IN THE SOURCE OF ATTRACTION

The response to the stimulus caused by the addition of a chemical to an attractant was measured by obtaining data on the 'rejection threshold' of the foraging population. In this account the term 'rejection threshold' is arbitrarily defined as the range of concentrations of the repellent which, when added to the standard attractant, produces a graded response extending from full acceptance to complete avoidance.

The response was measured when alternatives of the pure attractant were offered either alternating in space or in time.

Experimental lay-out

The chemotropic response was measured by counting the visits of foraging bees to dishes placed on a table in the greenhouse. The number of visits made to the pure attractant was compared with those made to the attractant containing a known concentration of the repellent.

The term 'visit' meant that a bee alighted on a dish and commenced feeding.

Various preliminary experiments were made to test different arrangements of the dishes which consisted of solid watch-glasses holding 5 ml. All patterns involving rectangular shapes were found to be unsuitable because the bees memorized the position of dishes, especially those at the corners, and any complicated pattern had to be avoided because of the difficulty of keeping a large number of dishes under constant observation. It was found that eight dishes (four containing the attractant and four the attractant with repellent) were the maximum that could be effectively observed by one person.

The following method of presenting the dishes to the bees was finally adopted:

A turn-table 1 ft. in diameter was geared to a small electric motor so that it made one revolution every 16 min. and the eight dishes were arranged around the edge.

This arrangement was found to be very satisfactory as it constantly changed the position of each dish relative to the hive, and the circular arrangement eliminated 'edge and corner effects'. It also tended to prevent the bees continually returning to one dish having remembered its position.

Experiment 1. *To test the reproducibility of the evaluation of rejection thresholds under greenhouse conditions*

It was considered necessary to determine the variability in the response of the population of foraging bees to a given chemical added to sugar solution.

Method. A large volume of 2M-sucrose solution in distilled water was prepared, and this constituted the standard attractant. This concentration was selected because it was readily acceptable to the bees and yet not so concentrated that it was liable to become viscous by evaporation of water on warm days.

Phenol was selected as a test chemical for this experiment, since, besides being a known bee repellent, it is fairly soluble in water and stable in aqueous solution. Using the stock 2M-sucrose solution, fourteen different concentrations of phenol were made up, ranging from 0·1 to 0·000001%, and each solution was labelled with a code number. This was considered desirable so as to ensure that the observer was not biased in his records by a knowledge of the concentration of the test solution. Each solution was tested separately on the turn-table against the standard sucrose solution, and the responses were measured by recording the total number of visits to the phenol solution when 50, 100 and 200 visits respectively had been made to the pure sucrose. Three complete tests were carried out for each solution over a period of 10 days.

The results are shown in Table 1, and the response to each concentration is expressed as an index of repellency. This index figure is obtained by expressing the number of visits to the phenol solution as a percentage of the number of visits to the pure sucrose and subtracting this figure from 100. Thus the final index figure is directly proportional to the stimulus of the repellent.

Experiment 2. *Repetition of Exp.* 1, *using an alternative technique which obviates errors in observation*

Butler, Finney & Shiele (1943) conducted experiments to determine the responses of the honey-bee to various chemicals, and they related the stimulus to the uptake of sucrose containing the chemical as compared with the uptake of pure sugar. The uptake of solution was measured volumetrically. This technique assumed that the bees when visiting a sugar solution normally drink a fairly constant amount of fluid. The actual experiments were carried out using the cells of a honeycomb.

The tests carried out in Exp. 1 were repeated and the response was measured by an adaptation of the above method. The differences of uptake of the various solutions were obtained by weighing before and after foraging had taken place. The dishes used in Exp. 1 were replaced by eight aluminium containers each holding 5 ml. These were filled with the respective solutions and exposed for varying periods in the greenhouse, the time depending on the activity of the bees. Where possible

Table 1. *Repellency indices at different concentrations; at three successive tests carried out over a period of ten days*

Concentration (%)	29. vi. 50–1. vii. 50 Repellency indices				3. vii. 50–5. vii. 50 Repellency indices				5. vii. 50–8. vii. 50 Repellency indices			
	When 50 visits made to sucrose	100 visits made to sucrose	100–200 visits made to sucrose	200 visits made to sucrose	When 50 visits made to sucrose	100 visits made to sucrose	100–200 visits made to sucrose	200 visits made to sucrose	When 50 visits made to sucrose	100 visits made to sucrose	100–200 visits made to sucrose	200 visits made to sucrose
0·1	100	100	100	100	100	100	100	100	100	100	100	100
0·05	100	100	100	100	100	100	100	100	100	100	100	100
0·025	98	98	99	98·5	100	98	99	98	94	100	96	98
0·010	82	86	84	85	100	97	98	97·5	100	98	99	98·5
0·005	62	57	65	61	58	87	80	83	88	54	50	52
0·001	28	55	73	64	42	53	60	56	42	41	45	43
0·0005	42	50	53	52	8	12	11	12	50	53	49	51
0·00025	12	30	41	35	10	10	34	22	41	51	55	53
0·0001	32	18	17	17	20	31	18	25	40	37	35	36
0·00005	40	29	20	25	72	52	63	57	24	29	10	20
0·00001	42	36	58	47	30	24	50	37	34	39	39	39
0·000005	54	38	27	32	50	40	43	42	46	24	38	31
0·0000025	12	0	−8	−4	4	−4	10	3	0	−1	−1	−1
0·000001	6	0	+6	+3	2	+2	−6	−2	6	+2	−11	−5
0·00000	5	−2	+3	+1	−5	−1	+2	+1	1	+6	−1	+3

a decrease in weight in the pure attractant approximately corresponding to the visits of two hundred bees in Exp. 1 was obtained. This method obviated any errors in observation, and the results only require to be corrected for evaporation. The results are shown in Table 2, and during the tests a number of counts of foraging bees visiting the pure sucrose were made so as to determine the natural variation in uptake per visit. These results are tabulated in Table 3.

Exps. 1 and 2 were successive tests carried out in the greenhouse with a fairly constant population of foraging bees extending over a period of 3 weeks. A visual inspection of the results shows that the response to the high concentration of phenol in all the tests was very constant. Both methods revealed an increase in variance with decrease in concentration, but the observational method used in the first experiment showed that from 0·000005 to 0·0000025 % there was a rapid falling off in the bees' response to the stimulus of the phenol. The fact that this falling off was not so clearly revealed by the weighing method is partly explained by the results shown in Table 3, which show that there is a normal variation in uptake when bees are foraging from a sucrose solution. The S.E. was calculated at ± 0·239 g. sucrose solution per hundred visits of the bee.

It would appear that the observational method gives a more accurate determination of the full range of concentrations involved in the rejection thresholds than is obtained by the weighing method, though the latter might prove useful where a rapid determination of approximate values was required.

There was no evidence of any definite trend in the results over the 3-week period which could suggest that the bees were becoming conditioned in their responses.

It was expected that the results would have revealed a relationship between concentration and response of a sigmoid nature, but this did not happen. In approximately the range 0·0001 to 0·00001 % a slight increase in repellency occurs; this is most marked in Table 1 but is also evident in Table 2.

A comparison of the indices for each experiment in Table 1 at the successive points of 50, 100 and 200 visits to the pure syrup indicates that where only the limits of the response are required, no advantage is obtained by continuing the test beyond the point when 50 visits have been made.

Experiment 3. *The determination of data relevant to the rejection thresholds of a number of chemicals, some of which are known insect repellents*

Using the method described in Exp. 1, information on the rejection threshold was determined for the following chemicals, viz. phenol, dimethylphthalate, acetic acid, Rutgers 612, isopropyl cinnamate and Teepol (a commercial detergent).

Insoluble oils such as dimethyl phthalate were emulsified using cyclohexylamine dodecylsulphate at a concentration of 0·03 % w/v. This was chosen as preliminary experiments indicated that some detergents such as Teepol were active repellents, whilst CHDS at this concentration did not elicit any response from the bees.

The results as shown in Table 4 indicate that at a concentration of 1 % all the compounds tested were equally unacceptable, and that as the concentration decreased the repellency of each solution also decreased. The results further show

Table 2. *Results of Exp. 2 corrected for evaporation*

w/v Concentration (%)	Wt. of 2 M-sucrose consumed	Wt. of sucrose + phenol consumed	$\frac{\text{Wt. of sucrose + phenol}}{\text{Wt. of sucrose}} = X$	Repellency index = 100 − (100 × X)
Date 10. vii. 50–12. vii. 50				
0·05	16·491	0·000	0·0	100
0·025	12·226	0·000	0·0	100
0·010	6·682	0·480	0·01	99
0·005	12·220	4·060	0·31	69
0·001	8·330	4·310	0·52	48
0·0005	9·980	5·380	0·53	47
0·00025	7·750	6·570	0·86	14
0·0001	10·710	8·880	0·83	17
0·00005	6·030	4·440	0·74	26
0·00001	9·926	8·910	0·90	10
0·000005	11·230	8·880	0·79	21
0·0000025	7·860	9·290	1·19	−19
0·000001	12·256	10·491	0·87	+13
0·000000	6·001	7·340	1·22	−22
Date 13. vii. 50–15. vii. 50				
0·05	11·290	0·000	0·0	100
0·025	14·100	0·000	0·0	100
0·010	15·670	1·070	0·06	94
0·005	15·845	4·930	0·31	69
0·001	12·718	4·780	0·37	63
0·0005	21·140	10·770	0·51	49
0·00025	6·670	4·080	0·61	39
0·0001	9·550	6·280	0·66	34
0·00005	10·230	6·280	0·62	38
0·00001	8·050	6·290	0·78	22
0·000005	10·330	12·590	1·21	−21
0·0000025	9·210	8·890	0·96	+ 4
0·000001	8·860	6·605	0·74	26
0·000000	6·120	5·280	0·87	13
Date 15. vii. 50–16. vii. 50				
0·05	9·250	0·000	0·0	100
0·025	14·142	0·000	0·0	100
0·010	6·780	0·250	0·03	97
0·005	7·195	2·050	0·28	72
0·001	6·215	2·140	0·34	66
0·0005	6·650	3·460	0·52	48
0·00025	8·340	3·105	0·37	63
0·0001	11·340	6·255	0·55	45
0·00005	7·750	4·730	0·61	39
0·00001	6·710	3·325	0·50	50
0·000005	12·350	7·740	0·63	37
0·0000025	8·720	6·680	0·78	22
0·000001	4·850	4·790	0·99	1
0·000000	10·340	9·980	0·96	4

Table 3. *Weights of 2 M-sucrose consumed by bees in 100 visits*

g.	g.	
4·12	4·14	Mean = 4·92
5·70	4·78	S.E. = ± 0·239 g.
5·05	5·62	
5·07		

that the range of concentrations included in the rejection threshold varied considerably with the different chemicals.

Isopropyl cinnamate appeared to become attractive at a dilution of 0·0025 %, but observations during the experiments suggested that emulsions of this type need very careful treatment as they tend to alter in form on dilution, and this may produce anomalous results.

Table 4

Chemical	Repellency index at the following concentrations (%)									
	1	0·1	0·05	0·01	0·005	0·0025	0·001	0·0001	0·00001	0·000001
Phenol	100	100	100	93	65	·	54	29	41	6
Acetic acid	100	61	47	46	2	−5	·	·	·	·
Rutgers 612	100	100	74	51	21	5	·	·	·	·
Dimethyl-phthalate	100	96	·	57	10	2	4	·	·	·
Teepol	100	100	94	80	61	58	39	10	2	·
Isopropyl cinnamate	100	95	·	68	16	−55	−6	·	·	·

All concentrations were volume/volume relationships.

Experiment 4. The evaluation of the threshold response when an alternative food source was available in time but not in space

In the previous experiments the bees were simultaneously offered two solutions of the attractant, one of which contained the repellent material, and it might be expected that if the pure attractant was removed, the bees would accept a much higher concentration of the repellent material.

The experimental method used in the previous experiments was adapted to investigate this effect. Metal covers were used to cover four of the dishes on the turn-table, and in this way it was arranged that the bees only had access to one solution (see Fig. 1). The number of visits to these dishes were recorded for a fixed time and the metal covers transferred to the other four dishes. The method is best illustrated by the following example:

No. of visits by bees in 5 min. to 2 M-sucrose	31
No. of visits by bees in 5 min. to 2 M-sucrose and 0·03 % phenol	10
No. of visits by bees in 5 min. to 2 M-sucrose	40
No. of visits by bees in 5 min. to 2 M-sucrose and 0·03 % phenol	16

The above technique was used to test the following chemicals: phenol, D.M.P., Rutgers 612, Teepol and acetic acid; the results are shown in Table 5.

The rejection thresholds indicated by this experiment are quite different from those in Exp. 3; all the concentrations involved are higher, though the various chemicals retain approximately the same order of effectiveness. In both experiments phenol was the most active repellent, and the lowest concentration involved in the rejection threshold changed from 0·000001 to 0·01 % when no alternative food source was present.

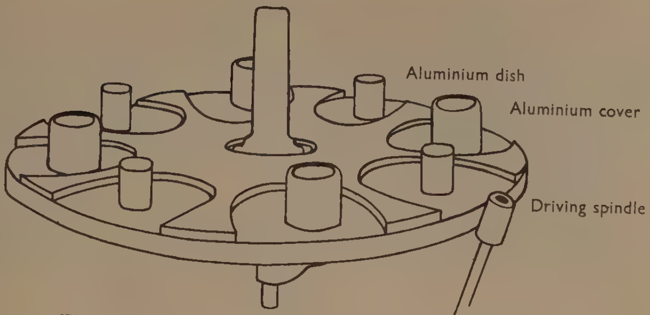

Fig. 1. Turn-table with dishes arranged as for Experiment 4.

Table 5

Repellent	Concentration (%)	No. of visits to pure sucrose (S)	No. of visits to sucrose and repellent ($S+R$)	R.I. $= 100 - \frac{S+R}{S} \times 100$
Acetic acid	0·8	71	0	100
	0·7	62	5	92
	0·6	58	26	55
	0·5	61	35	44
	0·4	53	35	34
	0·3	59	56	5
	0·2	68	69	0
Rutgers 612	0·2	52	0	100
	0·18	63	0	100
	0·125	58	15	74
	0·1	68	47	38
	0·09	53	45	15
	0·08	64	60	6
Dimethylphthalate	0·5	60	0	100
	0·1	49	5	90
	0·09	48	28	42
	0·08	72	43	41
	0·07	68	43	37
	0·06	73	47	36
	0·05	55	60	−9
Phenol	0·05	79	0	100
	0·04	77	4	95
	0·03	71	25	65
	0·02	67	63	6
	0·01	68	70	−1
Teepol	0·5	103	0	100
	0·4	33	3	94
	0·3	50	7	86
	0·2	56	1	98
	0·1	59	20	68
	0·09	47	23	49
	0·08	52	29	45
	0·07	61	37	40
	0·06	73	40	45
	0·05	80	65	19

Experiment 5. *The effect of varying the concentration of the attractant*

The last experiment was repeated using only phenol and acetic acid as test chemicals. These were made up in 1 M-sucrose solution, and this concentration was also used as the standard attractant. The bees did not forage as actively as they had done in the previous experiments, but the results as shown in Table 6 indicate yet a third set of figures relevant to the rejection threshold, lower in concentration than when the 2 M-sucrose was used.

Table 6. *Repellency index when solutions are made up in* 1 M-*sucrose*

	Concentration (%)	Repellency index calculated as in Table 5
Phenol	0·05	100
	0·02	77
	0·01	66
	0·009	37
	0·007	17
	0·006	2
Acetic acid	0·6	87
	0·5	69
	0·4	72
	0·3	70
	0·1	50
	0·09	37
	0·07	10
	0·06	8

Experiment 6. *To determine whether bees having once sampled a repellent solution are conditioned not to return*

Teepol, a commercial detergent, was found to have some repellent properties, and Exp. 3 showed that the concentration giving a repellency index of approximately 50 was determined to be 0·0025 %.

The eight dishes, four containing sucrose and Teepol and the other four with plain sucrose, were placed in the greenhouse. All the bees visiting the plain sucrose were marked white, and those visiting the repellent were marked red. Those visiting both solutions received red and white marks. This was continued for 3 hr. until about 150 bees had been marked. The dishes were refilled and all the bees' visits were recorded as red, white, red and white or unmarked (UM.). This was continued for 2 hr. and the results are shown in Table 7. The total number of visits to the plain sucrose solution is approximately twice the number made to the sucrose and Teepol, indicating an index of repellency of 58, which corresponds to the original figure at this concentration.

Table 7. *Repellency index as determined for* 0·0025 % *solution of Teepol* 56

Plain sucrose				Sucrose and Teepol 0·0025 %			
R.	W.	R.W.	UM.	R.	W.	R.W.	UM.
6	28	72	46	3	4	34	23
	Total	152			Total	64	

The visits made by the various groups of bees (red, white, red and white, and unmarked) to the two solutions follow the same distribution, though at different levels, with the exception of the bees which had only received a white mark. These had made twenty-eight visits to the plain sucrose and only four to the repellent. This result might be due to the sudden crowding of bees on to a particular dish during the experiment, in which case the discrepancy would have disappeared if the experiment had been continued longer.

The alternative explanation is that some of the bees were more prone either to avoid the repellent or to concentrate on the plain syrup, and if this was the case the response of the bees is not entirely uniform and some conditioning had taken place, though it would appear from this and previous experiments that such a variation in response by a small proportion of the bees did not seriously affect the reproducibility of the results.

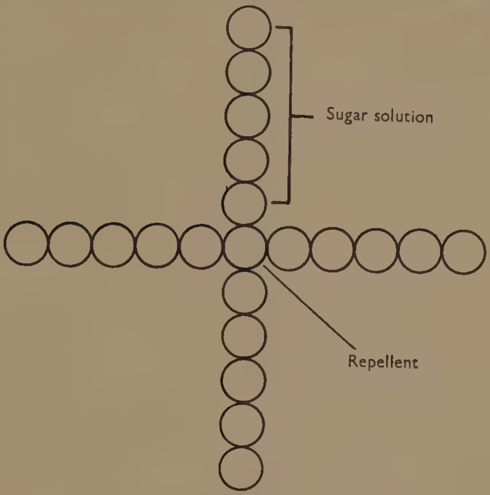

Fig. 2.

II. THE MEASUREMENT OF THE RESPONSE OF THE BEE TO A CHEMICAL IN THE VICINITY OF AN ATTRACTANT

Experimental methods

Twenty-one dishes, each having a capacity of 5 ml. and an external diameter of 2·5 cm., were arranged in the shape of a cross as shown in Fig. 2.

The repellent was placed in the central dish and the others were filled with a 2 M-sucrose solution. The dishes were placed on a tray on the training table in the greenhouse. The number of visits to each of the four groups of four dishes equidistant from the centre was counted. A bee would often drink at one dish and then at another of the same row—this would be counted as two visits. The fifth group of dishes, i.e. those at the end of each arm, used to remove 'edge' effects caused by their position at the end of the row, and visits to the group were not counted in the assay. The experiment was terminated when 100 visits had been made to the fourth group of dishes. The whole tray bearing the dishes was moved through 45° every 15 min. to avoid the bees remembering the position of any particular dish and returning to it.

Table 8. *Number of visits*

Concentration of cresol (%)	Dish no. 1	Dish no. 2	Dish no. 3	Dish no. 4
0·0	103	118	91	100
0·25	100	105	121	100
0·50	53	78	100	100
1·0	37	93	97	100
50·0	33	63	75	100
100	23	71	90	100

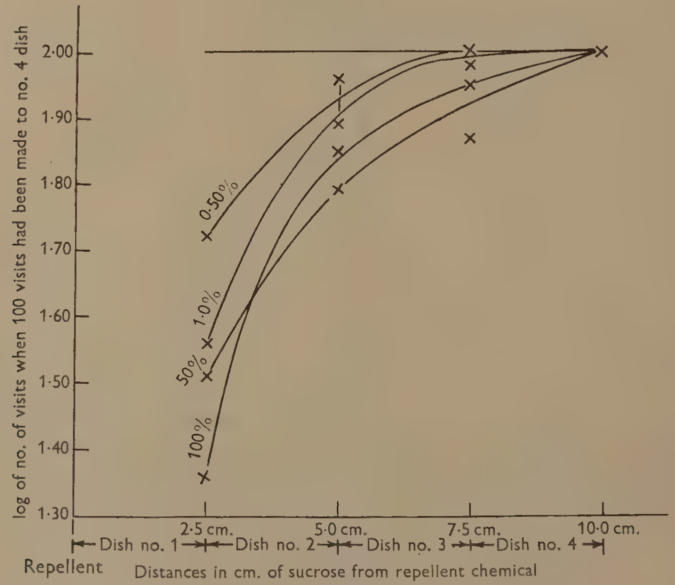

Fig. 3.

The results obtained from a series of tests using emulsions of various strengths of cresol (mixed isomers) in the central dish are shown in Table 8 and in Fig. 3. A preliminary test was first made without the repellent, as a control. The experiments were all done on the same day and took about 2 hr. each.

Fig. 3 shows that the bees' response to the stimulus varied with the concentration and that, assuming all the bees drank at the first group of dishes at the farthest point from the cresol, it appeared that until the concentration exceeded 0·25 % the bees showed no response to the presence of the stimulus at a greater distance than 2·5 cm., but as the concentration increased the distance over which a response was elicited also increased.

The experiment was repeated with other repellents, e.g. oil of citronella and creosote, and the results are shown in Table 9.

The tests indicate a possible technique for measuring this type of response, and it was felt that the replacement of the rows of dishes by a long narrow trough would allow for more accurate measurements to be made and would also remove a difficulty due to the fact that when the level of solution had been lowered in one dish it appeared to be more attractive to the foraging bees.

Table 9. *Visits to dishes*

Chemical	Concentration (%)	1	2	3	4
Oil of citronella	100	33	51	92	100
Oil of citronella	1	69	85	85	100
Creosote (crude)	1	35	75	76	100
Teepol (mixtures of sodium salts of secondary alkyl sulphates)	100	23	87	81	100
Isopropyl cinnamate	100	8	17	56	100

GENERAL DISCUSSION

The three different sets of data obtained during the experiments on the rejection thresholds of phenol and acetic acid are compared graphically in Fig. 4. The graphs indicate that the concentrations which completely repel all the bees were very similar under the three different experimental conditions, viz. with an alternative of pure sucrose in space, with an alternative of pure sucrose in time, (*a*) using 2 M-sucrose and (*b*) 1 M. When the last two conditions applied, both the curves approximate to a straight line. It is interesting to note that the three different conditions appear to exert a similar effect on the bees' responses to both chemicals. These variations in response, especially the increase in repellency shown with phenol over the range 0·0001–0·00001 % concentration, and also a similar trend being apparent for acetic acid, appear to be relevant to the problem mentioned by Dethier (1948) of whether gaseous mixtures act as repellent odours through the medium of localised olfactory receptors or as irritants through the medium of the more generalised common chemical sense.

For the purposes of this discussion we can define the olfactory, gustatory and common chemical senses as follows:

The olfactory sense is that stimulated by the chemical in gaseous form.

The gustatory sense is that stimulated by the chemical in solution.

The common chemical sense is that stimulated by chemicals acting as irritants and always produces an avoidance reaction. It must be mentioned here that there is some doubt as to whether, in the case of the honey-bee, it is permissible to consider the olfactory and gustatory senses separately (Butler 1949), and also that there are still wide gaps in our knowledge on chemoreception in insects.

In the experiments described in this paper it is likely that only the olfactory and common chemical senses were involved. The gustatory sense is only stimulated when actual contact with the test solution is made, and observations during the experiments indicated that nearly all the bees which alighted on the dishes drank some solution after inserting their proboscis and were thus counted. Very occasionally a bee would dip in its proboscis then fly away.

When the bees were offered a solution of the attractant which contained the repellent it may be assumed that at the lower concentrations of the latter only the olfactory sense organs would be stimulated. When the concentration of the repellent is progressively increased it is conceivable that a point is reached where the olfactory senses cease to be effective. (A parallel situation is commonly observed in humans who can only detect certain odours at low concentrations.) Further increases in concentration results in the stimulation of the common chemical senses by the repellent acting as an irritant.

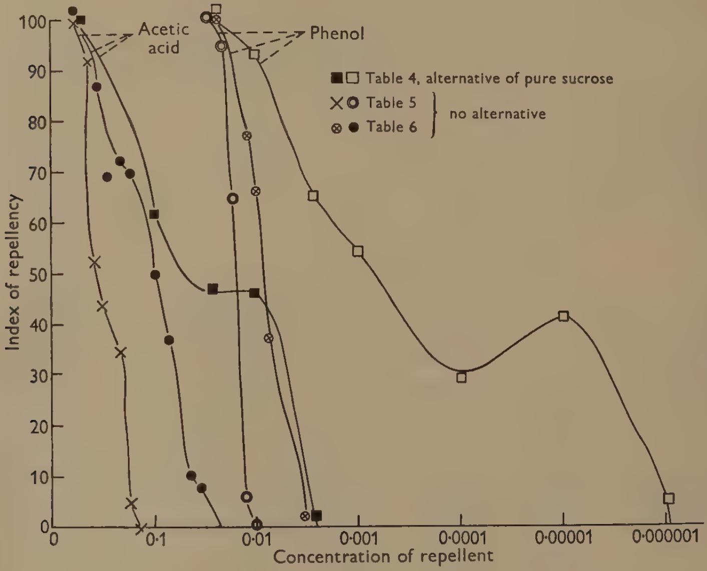

Fig. 4.

When a choice of solution was available (Exps. 1–3) the trends of the graphs for acetic acid and phenol may be interpreted as indicating the insect's response to two stimuli, one caused by the lower concentrations affecting the olfactory sense organs and the other at the higher concentrations causing an irritation of the common chemical sense. With phenol, and less markedly so with acetic acid, it would appear that with a progressive increase in concentration the olfactory sense ceases to be effective before the concentration is high enough for the chemical to act as an irritant. Hence the falling off in the response which occurs between 0·00001 and 0·0001 % phenol.

If the choice in space is removed (Exps. 4, 5) it would appear that the olfactory sense ceases to elicit a response, as there is now only a conflict between the stimulus inherent in the worker bee to collect food and the power of the chemical to act as an irritant and hence elicit an avoidance reaction. Under these circumstances it would be expected that the range of concentrations defining the rejection threshold would be much narrower and the experimental evidence confirms this expectation. In Exp. 5 when the strength of the attractant is decreased the phenol and acetic acid become more repellent but follow the same type of response curve. As in Exp. 4, the type of curves obtained when no choice is available support the hypothesis that under these conditions only one sense is being stimulated.

Other experiments not quoted here showed that in a large apiary when there was little food in the hives and no sources of nectar available in the field, higher concentrations of phenol than 0·025 % are accepted, but here the urge to collect food was probably much greater and there was an exceptionally large population of foraging bees.

SUMMARY

1. This paper is concerned, first, with the development of a technique for maintaining a population of honey-bees (*Apis mellifera*) under conditions where it does not have access to naturally secreted nectar, but is allowed free flying conditions, and secondly with the application of this technique to the study of the chemotropic responses of bees to repellent chemicals.

2. Two types of stimuli are considered:

(*a*) The effect of the addition of the repellent to an attractant (sucrose solution).

(*b*) The effect of the repellent in the vapour phase in the vicinity of the attractant.

In (*a*) techniques are described for measuring the response with the standard attractant alternating in time and space.

3. Data collected on the rejection thresholds of acetic acid and phenol showed that whilst little variation in response occurred at the higher concentrations of the repellent, the offering of the standard attractant alternating in time as opposed to space greatly modified the response. Tests were carried out to determine the reproducibility of the results.

4. It would appear that for a repellent chemical to be capable of effectively reducing the number of visits to a source of food, it must be capable of irritating the common chemical senses of the bee.

I am indebted to the Agricultural Research Council for a grant which has made this work possible; also to Dr C. G. Butler and Dr C. Potter of Rothamsted Experimental Station for helpful advice and criticism. I am especially grateful to Mrs W. Heatherington, B.Sc., and Miss M. Burgoyne for giving valuable assistance with the experimental work.

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DIFFERENTIAL EFFECTS OF DIRECT CURRENT ON INSECT GANGLIA

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(*Received* 24 *November* 1951)

(With Plate 16 and Six Text-figures)

INTRODUCTION

A transverse section through any insect ganglion discloses a mass of densely interwoven dendritic connexions forming a central neuropile surrounded by a peripheral layer of cell bodies. Histologically these ganglia are extremely complicated, and it is unlikely that a complete knowledge of the connexions involved in reflex activities will be obtained for many years. In fact, it may be impossible to construct detailed maps of nervous pathways in such ganglia on account of their organization being essentially different both morphologically and physiologically from systems which have been successfully analysed in terms of separate pathways conducting discrete nervous impulses. Herrick (1929) pointed out this distinction between a system of definite nervous tracts and that of a neuropile in which the whole fabric seems equipotential and with little possibility of impulses being confined to particular reflex circuits. In this paper attention is given to the possible importance of continuous changes in electrical potential in addition to the discontinuous changes which have been studied so much in neurophysiology. While only future research can determine the importance of such continuous changes in the neuropile, many observations indicate that they are involved in the functioning of this and other parts of nervous systems.

Many workers, such as Hermann (1885), Loeb & Maxwell (1896) and Miller (1907), have studied the phenomenon of galvanotropism in Crustacea and suggested that the effects were produced by stimulation of the central nervous system. They showed that when the animals were placed in an electric field they orientated themselves with respect to the direction of current flow. This orientation was brought about by a differential excitation of the body and limb musculature. In *Palaeomonetes* Loeb & Maxwell showed that the abdomen and fifth limb pair were flexed and the third pair of limbs extended when the head was towards the cathode (ascending current). Reversal of the current produced the opposite effects. With transverse currents the legs on the negative side extended and those facing the anode were flexed. To explain these observations, Loeb & Maxwell constructed a theory which was based on the assumption that the cell bodies of the flexor and extensor neurones were orientated differently in the ganglia. Miller (1907) agreed that the effect on the central nervous system was important, but suggested that direct stimulation of the motor nerves was also involved. Loeb & Maxwell emphasized that the differential effects were only observed with moderately strong currents; at higher intensities all the muscles were excited. Scheminzky (1943) and others have continued this study using strong currents. They have shown that with ascending currents all the muscles become active in a state of 'galvanic cramp', and with descending currents the animals become paralysed in 'galvanonarcosis'. Scheminzky (1936) observed these phenomena in many invertebrates and considers that his experiments indicate a 'functional polarity in the central nervous system'.

Although many early workers on galvanotropism had suggested that the direct currents stimulated the central nervous system, very few experiments were carried out in which polarization was applied directly to ganglia. Miller (1907) describes observations on the rhythmical contractions of the anal sphincter in the crayfish when the nerve cord was stimulated. These contractions were activated by ascending currents and depressed by descending currents, even when the ventral nerve cord was reduced to a single abdominal ganglion, but they disappeared on extirpating this structure. It was concluded that ascending currents stimulate the central nervous system and descending currents depress. Moore (1923) showed that direct currents passed transversely through an earthworm induced movement of the two ends towards the cathode. This U-shape was not obtained if the nerve cord was cut on either side of the stimulated region because the effects were not present beyond the incisions. Longitudinal currents with the head negative produced lengthening of the worm as a result of the contraction of the circular muscles, while the longitudinal muscles were excited by descending currents. Gray & Lissmann (1938) were unable to observe this reciprocal effect, but found that descending currents (head positive) encouraged peristalsis and the animal became quiescent when the current was reversed. Moore (1945) points out that spontaneous peristalsis may be superimposed on the tonic effect, and he suspended his animals in $\frac{1}{4}$ % agar to reduce this complication. He also observed the transmission of these differential effects from one part of the cord to another.

In a critical paper, Halbisch (1940) has produced convincing evidence in support of these observations of Moore. Halbisch investigated the problem in the stretched animal and found that the result of applying direct currents varied with the weight of the lever. The differential effects were only found in the majority of cases when he used a light lever. He pointed out the significant fact that the result varied even for a given individual, showing that although there is a definite functional polarity in the central nervous system, this is not constant. His results also show that the pattern of peripheral stimulation, depending on the stretch of the worm, is of great importance and exerts a marked effect despite the enforcement of a definite polarization within the central nervous system.

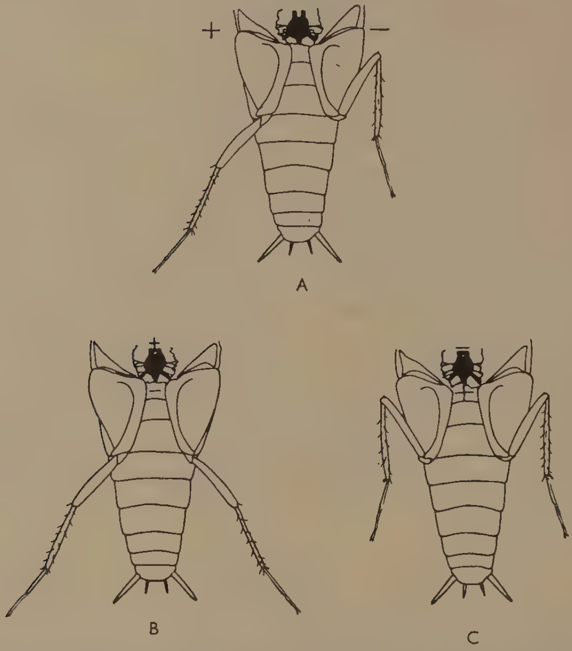
Auger & Fessard (1928) showed that direct-current stimulation of the ventral ganglia in crickets resulted in rhythmic movements of the elytra when the cathode was placed on the ganglia. In a later paper (Auger & Fessard, 1929) they record some excitation at the commencement of anodal stimulation. Increasing negativity of the ganglion produced a rise in frequency of the elytral movements which were always of the same size. More recently (Auger & Fessard, 1935) they have demonstrated that rhythmic flexor movements could be produced in a single cockchafer leg isolated with its ganglion. The electromyogram consisted of grouped discharges to the flexor muscle. Cathodal stimulation of the ganglion increased the frequency of these discharges and of the impulses in each group. Anodal stimulation depressed this activity. Pringle (1946), passing small polarizing currents through millipedes, obtained reciprocal effects with different directions of current flow. Descending currents produced tonic flexion of the legs, while ascending currents gave tonic extension. At certain current strengths he observed rhythmic movements of the legs, the frequency depending on the current intensity.

The most recent work in this field derives from the observation by Barron & Matthews (1938) that vertebrate motor neurones respond to a constant current by a rhythmical discharge of impulses. The frequency increases with the intensity of the applied current when the electrode on the cord is negative to one place on the ventral root. Reversal of the current inhibits any resting discharge. Skoglund (1947*a*) has recently published the results of his work on cats in which he passed small direct currents through the spinal cord in opposite directions and was able to record reciprocal effects in antagonistic muscles. With transverse currents, extension predominated on the positive side and flexion on the side of the negative electrode. Ascending currents stimulated flexion, and with descending currents the extensor muscles were brought into activity. He was able to obtain similar differential excitation of flexor and extensor motor neurones by using unipolar stimulation of the medulla (1947*b*). Bernhard, Skoglund & Therman (1947) were able to obtain monophasic ventral root potentials by lengthwise polarization of the cord. The sign of this potential depended upon the direction of current flow. Bernhard & Skoglund (1947) obtained the same result in response to medullary stimulation.

PROCEDURE

In the preliminary experiments, in which movements of the legs were observed, the decapitated cockroach (*Periplaneta americana*) was dissected from the dorsal surface and the alimentary canal carefully removed. The metathoracic ganglion was exposed and isolated from the rest of the ventral nerve cord. The insect was suspended by hooks from its anterior and posterior ends, so that the legs were completely free. In the experiments in which recordings were made of the nerve and muscle action potentials, the insect was pinned ventral side uppermost and the metathoracic ganglion exposed by dissecting off the overlying sclerites. Many of the tracheae were left intact during these dissections, as this was found to improve the condition of the ganglion.

Direct currents were applied from a stimulator similar to that used by Skoglund (1942). It was fitted with an output valve of high internal resistance in order to minimize the effects of changes in the resistance of electrodes and tissues. The stimulating electrodes were chloride-coated silver wires covered with a wick, one strand of which was drawn out and applied to the preparation. The wick and preparation were bathed in insect Ringer (Pringle, 1938), but the volume of fluid about the ganglion was reduced before stimulation. Nevertheless, the current paths were not entirely restricted to the ganglia, and the intensity recorded by a microammeter in series with the electrodes is much higher than the current actually passing through the ganglion. The recording electrodes were of platinum wire hooked under the nerve or inserted into the muscle. Condenser-coupled amplifiers with balanced input stages were used and the signals recorded on a Cossor double-beam oscilloscope.



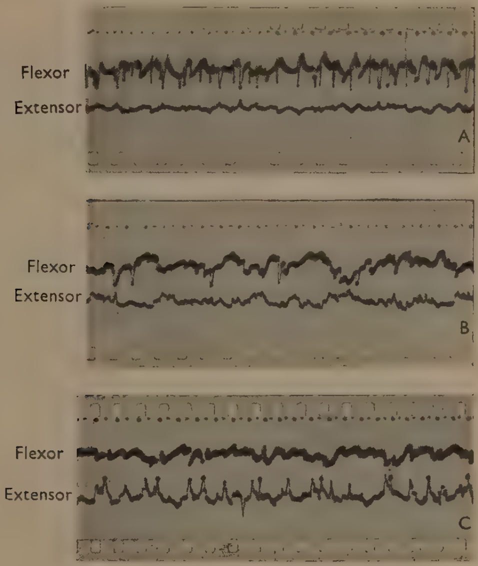
Text-fig. 1. Diagrams illustrating the reciprocal effects produced on passing small polarizing currents through the intact metathoracic ganglion of the cockroach. A, transverse current; B, descending current; C, ascending current.

RESULTS

(1) *Stimulation of the intact metathoracic ganglion*

The passage of small constant currents transversely through the metathoracic ganglion of a suspended cockroach produces changes in the flexor-extensor balance of the leg joints. The leg on the negative side tends to flex, while the contralateral leg extends (Text-fig. 1). At higher intensities (100 μA.) all the muscles are excited and both legs become rigidly extended. When the current is passed longitudinally, the legs on both sides of the body respond in the same way, but again the type of movement depends upon the direction of the polarizing current. With ascending currents (head negative) both legs flex, while descending currents encourage extension at all the joints. In some preparations a tendency to carry out rhythmical movements in response to the direct currents was observed, but the tonic effects predominate.

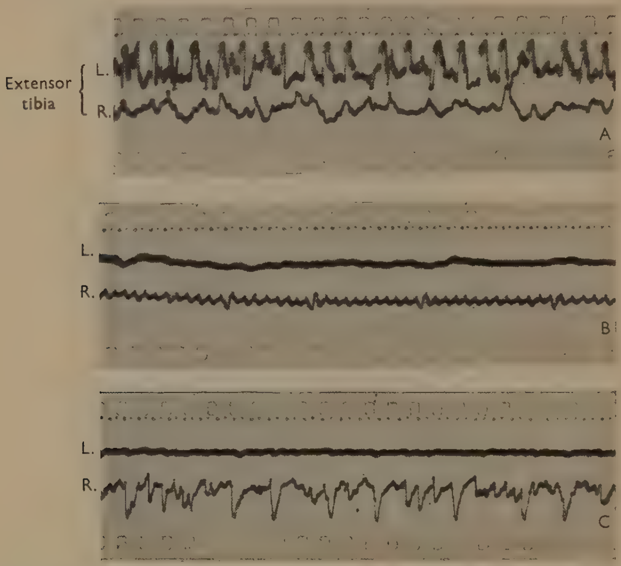
By recording the action potentials in the flexor and extensor trochanteris muscles, it was shown (Text-fig. 2) that these movements are brought about by a true differential excitation of antagonistic muscles. Care must be taken in placing the two pairs of electrodes as far apart as possible in order to restrict the pick-up of impulses from the antagonistic muscle. This is often difficult because the muscles are contained within such a restricted space. On passing the current across the ganglion, with the negative electrode on the side from which recordings are being made, several motor fibres to the flexor trochanteris are stimulated (A) and this muscle contracts. If the direction of the current is reversed, the frequency of these impulses decreases and some of the fibres drop out, while at the same time the size of the slow response of the extensor muscle increases (B). Further increase of the current intensity in this direction brings about more marked extension of the trochanter (C).



Text-fig. 2. Electrical records from the flexor and extensor trochanteris muscles of the left hind-leg during the passage of direct currents transversely through the metathoracic ganglion. A, 50 μA. negative electrode on the left side; B, 10 μA. positive electrode on the left side; C, 40 μA. positive electrode on the left side. The time-marker indicates 10 milliseconds.

If the activity of the extensor tibia muscle in both legs is recorded, similar reciprocal effects are obtained (Text-fig. 3). When the positive electrode is on the left side of the ganglion, the extensor tibia on this side is brought into activity and the contralateral one inhibited (A). Reversal of the current inhibits this activity in the left extensors and brings the slow fibre of the right extensor tibia into marked activity (B). The third record (C) shows the effect of further increasing the intensity of this direction of polarizing current; the response is stronger as more motor fibres are excited.

When the motor fibres are spontaneously discharging, augmentation or depression of the frequency can be brought about by the appropriate direction of current. In these experiments the current was gradually increased to threshold and supra-threshold values and recordings made at the particular intensity. Sometimes the decrease of a current in one direction initiates the same response that is obtained by an increase in the intensity of a current in the opposite direction. This effect is even more marked when the current flow is suddenly broken. Another interesting observation is the reversal of the normal effect at low intensities, the normal effect being found on raising the current intensity. For instance, on gradually increasing the strength of a transverse current, excitation of the extensor may take place on the negative side, but this is inhibited as the slow fibre of the flexor responds. Similarly, before ascending currents produce their usual excitation of the flexor muscles, the extensor may be active and is later inhibited as the normal response occurs. In these preparations where excitation of the extensor occurs on the cathodal side, the threshold is always lower than that of the slow flexor response. Hence the potential level within the ganglion required to produce this effect will be positive to that producing flexion just as in the majority of preparations.

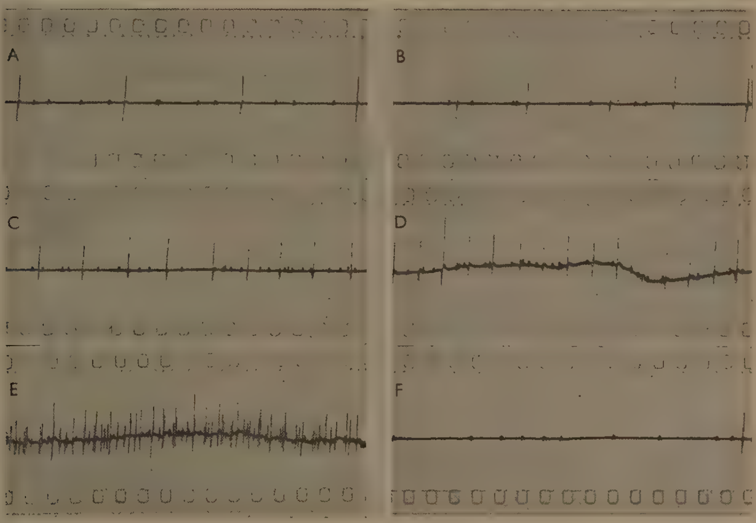


Text-fig. 3. Reciprocal effects in the extensor tibia muscle of the left and right hind-legs produced when direct currents are passed transversely through the metathoracic ganglion. A, 50 μA. positive electrode on the left side; B, 20 μA. negative electrode on the left side; C, 50 μA. negative electrode on the left side. The time-marker indicates 10 milliseconds.

These conclusions are based on the behaviour of a large number of preparations, which varied in the degree to which the reciprocal effects were obtained. An effect on the extensor-flexor balance was always present, and its nature largely depended upon the direction of the polarizing current.

(2) *Preparations with a single nerve attached to the metathoracic ganglion*

The innervation of the muscles in the third leg of the cockroach was determined by Pringle (1939), who made use of the stimulating action of nicotine on the ganglion. The major part of his description has been confirmed by tracing the course of the nerves stained intra-vitally with methylene blue. The distribution of the motor fibres is such that nerve III*b* chiefly innervates extensor muscles while nerve V innervates flexor muscles of the trochanter and tibia. A preparation with one of these nerves alone attached to the ganglion provides an opportunity to study the behaviour of the extensor and flexor systems separately.



Text-fig. 4. Records of the impulses in nerve V when polarizing currents are applied between the metathoracic ganglion and this nerve. A, resting discharge; B–E, increasing intensities (2, 5, 10 and 20 μA.) of current raise the frequency of this discharge when the negative electrode is on the ganglion; F, decrease in the frequency of the resting discharge with the positive electrode on the ganglion (5 μA.). The time-marker indicates 10 milliseconds.

One stimulating electrode was placed on the ganglion and the other on the single nerve, all other nerves being severed. The records reproduced in Text-fig. 4 and Pl. 16, fig. 1, were obtained from such preparations by amplifying the impulses led off from platinum electrodes hooked under the nerve. With nerve V attached to the ganglion any spontaneous discharge (A) is inhibited by a descending polarizing current (electrode on the ganglion positive) (F). When the negative electrode is on the ganglion the discharge increases in frequency as the current intensity increases (B–E). It can be seen that several motor fibres are being excited in exactly the same way.

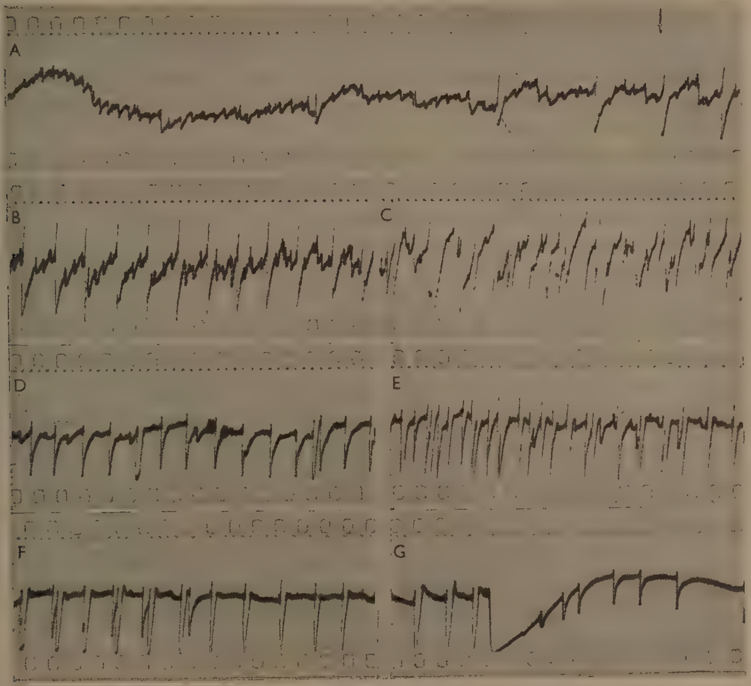
The corresponding experiments carried out with only nerve III*b* attached to the ganglion yield substantially the same result (Pl. 16, fig. 1). With ascending currents (B–F) the frequency of the resting discharge (A) increases gradually as the current intensity is raised and a larger fibre starts to fire off (C). The frequency increases steadily, but at higher intensities (F) the larger impulses, which have been discharging at a lower frequency than the original ones, fall off in frequency. Positivity of the electrode on the ganglion (H) inhibits the spikes of the resting discharge (G), but a further increase in this current (I) produces a new response. This consists of bursts of small impulses which fall off in frequency during each burst. It is characteristic of some preparations that a stage of intermittent discharges precedes complete inhibition of a resting discharge by a descending current.

Similar results are obtained when the recording electrodes are placed in the muscles supplied by these nerves. Furthermore, such recordings are of considerable value in studies of the neuromuscular mechanism of insects because the different motor fibres are excited in their functional order, i.e. the slow fibres are excited at lower thresholds than the motor fibres which bring about faster contractions of the muscle. Records are shown (Text-fig. 5) from the flexor tibia muscle during a typical experiment in which ascending currents were applied between nerve V and the metathoracic ganglion. At least three different electrical responses associated with contraction of the muscle are clearly visible. Small potentials ($100\,\mu$V.) showing gradual facilitation (A) are obtained at low intensities, and this is evidently the slow fibre response of Pringle (1939). The frequency of this and subsequent muscle potentials may be graded by variations in the current intensity. The second type of potentials are initially greater than the slow response and soon reach their maximum height (B and D). At higher current intensity ($15\,\mu$A.) very large potentials are brought into action (C) and are more readily visible at lower gains (D–F). The two larger types of potential are clearly distinguishable in the last two records which are respectively near the beginning and at the end of a period of direct-current stimulation. The marked after-discharge in the medium-sized response following prolonged activity in the largest system is typical of many preparations. The slow system frequently behaves in this way following the activity of one of the larger responses. These phenomena precisely parallel the patterns of electrical activity which have been recorded from this muscle during normal activity (Hughes, 1952).

Thus, in all the experiments in which a direct current was applied between the ganglion and a single nerve, there has been no unequivocal excitation of motor fibres when the electrode on the ganglion was positive. This strongly suggests that both the flexor and extensor motor neurones are excited in the same way. Such a result was unexpected in the light of the behaviour of the intact ganglion preparations and possible interpretations will be discussed below.

(3) *The effects produced by the passage of polarizing currents through other ganglia*

Similar experiments to those described above performed with the mesothoracic and prothoracic ganglia yielded substantially the same results. Transmission of the reciprocal effects from one ganglion to another is also occasionally found. For instance, the passage of transverse currents through the mesothoracic ganglion frequently affects the flexor-extensor balance of the hind-legs provided the commissures are intact. There is often a well-marked effect upon this balance when constant currents are passed through the suboesophageal ganglion. The antagonistic



Text-fig. 5. Recordings from the flexor tibia muscle during the passage of ascending currents of increasing intensity between the ganglion and nerve V. A, B and C are at higher gain than records D–G. A, 5 μA. Slow fibre response and also six larger potentials showing more rapid facilitation. B, 10 μA. Slow fibre responses have increased in frequency and are now more facilitated. Frequency and facilitation of second type response have also increased. C, 20 μA. More complicated record with both previous types of potential and probably even larger ones. D, 15 μA. At lower gain the slow response is scarcely visible but there are clearly two types of larger potential. E, 20 μA. The largest potential type of a twitch-producing system increases in frequency. F, 17 μA. Shortly after the commencement of polarization the two large types of potential are present, but as stimulation is continued the larger response predominates. G, end of polarization as in F. The largest potentials cease abruptly and there is a marked after-discharge in the medium-sized potentials. The time-marker indicates 10 milliseconds.

muscles may be set alternating rhythmically by a very small current, and as descending currents are increased the flexor activity becomes predominant.

Polarization of the suboesophageal ganglion also produced very definite reciprocal effects upon the maxillary palps whose muscles are innervated from this ganglion (Pringle, 1938). The two extrinsic muscles are differentially excited by longitudinal currents and bring about promotor movements when they are descending. Ascending currents produce the opposite effect, and some of the intrinsic muscles also contract and flex the palp joints. Transverse currents likewise induce tonic movements, promotion on the negative side and remotion on the anodal side. These reciprocal effects disappear at current intensities of 15 μA. or more, when all the muscles are excited.

Ascending from synapses in the last abdominal ganglion there are giant fibres, six on each side, which are excited by stimulation of fine hairs on the anal cerci by air movements (Pumphrey & Rawdon-Smith, 1937; Roeder, 1948). Recording electrodes placed on the ventral cord readily pick up the large action potentials of these fibres. Direct currents produce excitatory and inhibitory effects when applied between the ganglion and ventral nerve cord (Pl. 16, fig. 2). Cathodal stimulation of the ganglion is usually exciting and anodal depressing, but the reverse effects have been recorded in some preparations. In many preparations it has been observed that an increase in the polarizing current produces an initial high-frequency burst in the giant fibres which is then replaced by a discharge of lower frequency as the current continues. The frequency both of this initial burst and of the steady discharge increases as the current intensity is raised. This observation is reminiscent of the 'adaptation' of synaptic transmission through this ganglion described by Pumphrey & Rawdon-Smith. They found that the responses of the giant fibres decreased with repetitive submaximal stimulation, but this was restored on increasing the magnitude or frequency of the presynaptic stimuli. They interpreted this observation in terms of a rise in the threshold of the adapted synapse, which is also indicated by the results obtained with direct-current stimulation.

DISCUSSION

There can be little doubt that the passage of direct currents through ganglia of the cockroach nerve cord produces well-marked excitatory and inhibitory effects. The question arises as to the nature of the structures directly stimulated by this polarization. The repetitive discharge of nerve fibres to direct-current stimulation is well known in Crustacea, but there is a complete absence of studies on this phenomenon in insects. It has been observed during the present research that the nerve axons are stimulated at high current intensities, but their discharge is of short duration and rapidly falls off in frequency, whereas many of the responses described above persist for a minute or more. If the electrodes were displaced laterally during longitudinal stimulation of the ganglion it was found that the excitatory effects of the current decreased rapidly with distance from the ganglion. As the current lines would be passing more through the nerves and less through the ganglion under these conditions, this observation suggests that the constant current stimulates some structure within the ganglion. Further evidence for this suggestion is provided by experiments in which nicotine was applied to the ganglion and brought about a complete abolition of the reciprocal and long-lasting effects described above. It is well known that following an initial excitatory effect, this drug depresses the synapses and cell bodies of ganglia while the nerves leaving the ganglia remain excitable.

The lower threshold of the slow-fibre response to a given muscle compared with that of the quick fibres is further evidence that a part of the normal central mechanism is being stimulated. Stimulation of the nerves peripherally excites the quick fibres at a lower threshold than the slow fibre (Pringle, 1939).

The results obtained on stimulating the last abdominal ganglion are also relevant to this problem of the site at which these currents produce their effects in insect ganglia. The similarity between the 'adaptation' observed by Pumphrey & Rawdon-Smith (1937) and the results of direct-current stimulation suggests that it is the synaptic region which is sensitive to polarization and not the cell bodies.

It has been shown that motor fibres to extensor and flexor muscles are differentially excited by currents of opposite direction in the intact preparation; two alternative hypotheses may be suggested to account for this phenomenon:

(i) There is an intrinsic difference between the response of the flexor and extensor collaterals within the neuropile to positive and negative currents.

(ii) Differences in the spatial distribution of the motor neurones or of their connexions within the ganglion account for the differential excitations.

The first hypothesis is not supported by the results of experiments in which only single nerves containing either flexor or extensor nerve fibres remained attached to the ganglion. No definite excitatory effect was observed in such experiments when the electrode on the ganglion was positive to the one on the nerve; some slight responses of the type shown in Pl. 16, fig. 1, are the only exceptions to this. This result is in direct contrast to Skoglund's observations on cats, but it is in agreement with the results of Barron & Matthews. These workers supposed that it was the cell bodies of the motor neurones that were stimulated by the constant currents, and such is probably the case in vertebrates where the cell body is the site of many synaptic knobs and plays an important role in reflex processes. In insects and other invertebrates, however, the cell bodies do not play such a direct role but are aggregated at the ganglion surface and send collateral branches into the central neuropile where all synaptic connexions occur. It is possible, therefore, that the low intensities of direct current are not stimulating the motor neurones directly but are influencing the discharge of impulses from their collaterals within the neuropile and that the cell bodies are only excited at higher intensities.

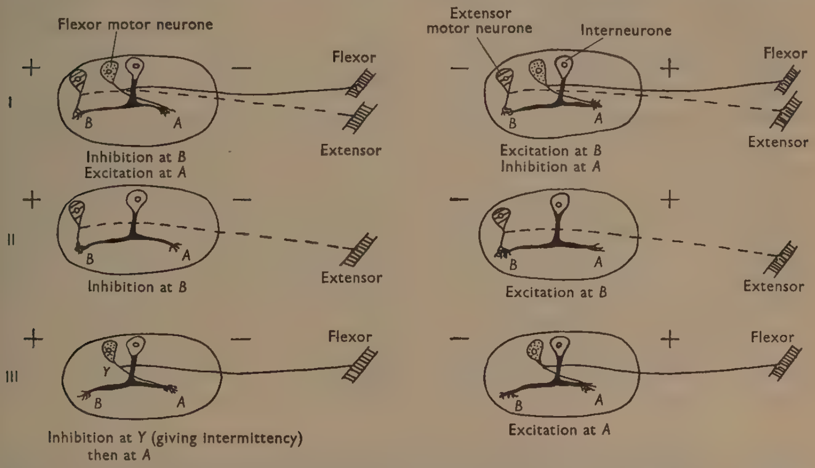
Skoglund (1947*a*) recognized the two alternatives considered above, and although he did not reach a definite conclusion, he inclined towards the one postulating different inherent properties. In this question he was influenced by investigations (von Euler & Skoglund, 1947) showing that different nerve fibres vary in their threshold to ascending and descending currents. These experiments are open to the criticism that the region of excitation was not identical for the two directions of current and this might account for the differences in threshold observed. The many workers (Arvanitaki, 1938; Fessard, 1936; Hodgkin, 1948) who have investigated the stimulation of crustacean nerve fibres with direct currents have not been concerned with the direction of the current with respect to the orientation of the fibres in the animal, and do not record any observations on this subject. In favour of a different distribution of flexor and extensor motor neurones are the results obtained by Aström (1947) by stimulating the cord with very finely pointed electrodes and mapping out the distribution of the thresholds of the antagonistic muscles.

The construction of a hypothesis with a definite histological basis is impossible at the present stage because the connexions within the cockroach ganglia are unknown. There are many interneurones in insect ganglia (Zawarzin, 1924), however, and it is reasonable to suppose that some of these connect the endings of antagonistic motor fibres within the neuropile. Such internuncials need not conduct impulses in the normal way but might function, in part, by electrotonic conduction. This is not improbable because such a property of insect nerve fibres has been observed by Parry (1947); Brooks & Eccles (1947) suggested a similar mechanism in the Golgi cells of the vertebrate spinal cord. Further histological studies of insect ganglia may disclose structures of a relatively large diameter which might conduct in this fashion. It is possible that polarizing currents passed through the ganglion influence the gradient between the flexor and extensor ends of these internuncials. Thus, the endings at the negative end of the internuncials will be stimulated and those at the positive end inhibited, the effects being reversed when the current direction is changed. This hypothesis suggests that the internuncial connexions between flexor and extensor pools within the ganglion have a definite orientation which has longitudinal and transverse components. Text-fig. 6 shows very diagrammatically how such a hypothesis might explain the phenomena described above.

Normally, the balance between flexor and extensor pools is regulated by impulses reaching these centres from sensory nerve fibres and from the other ganglia, chiefly the thoracic and suboesophageal. The effect of the latter upon the flexor-extensor balance of the thoracic ganglia is distinctly shown by the increased tone of the extensor trochanteris and other depressor muscles in a decapitated insect. Polarization of the suboesophageal ganglion has also been shown to influence the relative intensity of excitations reaching the flexor and extensor pools of the metathoracic ganglion. When there is only a single nerve attached to the ganglion there will be considerable disruption of this normal balance because of the absence of the normal pattern of impulses from other ganglia and sense organs. It seems probable that the balance of the internuncial system is so upset that the direct currents no longer influence this system but excite the motor neurone collaterals directly or maybe the cell bodies themselves (Text-fig. 6).

The occurrence of intermittent discharges in some preparations may be due to the presence of an anodal block at Y. Thus, if this became positive with respect to the synaptic endings (A), interruption of regular discharges from this region might occur.

The significance of reversed reciprocity at very low intensities in some intact preparations is difficult to assess but some analogies are of interest. Recent work of Wyss (1948) and Bernhard & Skoglund (1942) has confirmed the earlier observation of Fröhlich (1909) that stimulation of the same afferent pathway, which normally excites a given reflex, may produce inhibition at higher frequencies. Bernhard & Skoglund showed that stimulation at low frequencies might be inhibitory, but an increase in frequency caused a subsidence of this effect and excitation occurred. If we accept an electrical concept of synaptic transmission which supposes that an increase in frequency of the afferent inflow is equivalent to an increased depolarization at the central synapses, then this is being simulated by the increase in intensity of applied direct current. The similarity is striking and adds further confirmation to the supposition that these currents excite a part of the normal functioning system. The theory of central inhibition developed by Brooks & Eccles (1947) provides a mechanism whereby excitation of the same structure can produce different effects depending on the intensity of an electrotonic change in the central nervous system.



Text-fig. 6. Diagrams to show the suggested conditions of excitation and inhibition in the neuropile during the passage of direct currents through a thoracic ganglion of the cockroach. I, intact ganglion; II, nerve III*b* alone attached to the ganglion; III, nerve V alone attached to the ganglion.

CONCLUSIONS

From this study there emerges a picture of the possible way in which neuropiles may function. This envisages the neuropile as a three-dimensional distribution of potential fields which are continually modified by the action of a changing pattern of impulses from the peripheral sense organs. The endings of the motor nerve fibres in the neuropile mass may detect the potential level within their particular region just as a sense organ communicates information about specific parts of the peripheral field (cp. Matthews in Pantin, 1937). The central pattern will be altered by fluctuations in any part of the peripheral inflow, but the extent of this modification will vary according to the spatial relations of the central connexions for any particular fibre. A system such as this is more capable of definition where there are discrete ganglia as in the cockroach, but there is no *a priori* reason why a similar system should not operate in the spinal cord of a vertebrate. The experiments described in this paper have shown how the superposition of given directions of polarization on insect ganglia can alter the reciprocal relations of pairs of antagonists, and it is supposed that these effects are due to distortions of the neuropile field. Apart from such observations there is little evidence to support the above hypothesis, and many obvious difficulties are involved in the direct measurement of any distribution of potential levels within neuropiles.

The conception of neuropile function outlined above lays stress upon the importance of considering central nervous patterns as a whole. Given portions of the peripheral inflow are considered to modify the total distribution of potential levels rather than their influence being restricted to a small number of neurones. Recent work on both the mechanics (Gray, 1944) and neuromuscular co-ordination of animal movements (Gray & Lissmann, 1946; Hughes, 1952) has emphasized the importance of considering the skeletal, muscular and nervous systems of the animal as a whole rather than in isolated parts. It is to be hoped that some synthesis may emerge in the future of the results of years of a causal-analytical approach by many workers on problems of nervous co-ordination, and that in this synthesis the essential wholeness of the organism in all its activities will be stressed.

SUMMARY

1. The passage of small direct currents transversely through the intact thoracic ganglia of the cockroach affects the flexor-extensor balance of the legs in such a way that the leg on the negative side flexes while that on the positive side extends.

2. With longitudinal stimulation, ascending currents bring about flexion of both legs and descending currents encourage extension.

3. Preparations in which only a single nerve is attached to the ganglion do not show these differential effects when the polarizing current is applied between ganglion and nerve. The negative electrode excites when it is on the ganglion, and the positive inhibits irrespective of whether the nerve supplies flexor or extensor muscles. Intermittent discharge of impulses may occur before the spontaneous discharge is completely inhibited when the positive electrode is on the ganglion.

4. At least three different responses have been recorded from some of the leg muscles under these conditions and they are excited in a 'functional order'. That is, the 'slow' response accompanied by a tonic contraction occurs at a lower threshold than the 'faster' twitch systems.

5. A hypothesis is put forward to interpret these apparently contradictory results. It is suggested that the polarizing current influences the gradient of potential along oriented internuncials which are stimulated at low threshold in the

intact preparation. These internuncials connect regions of the neuropile containing flexor and extensor synaptic endings.

I should like to thank Mr. J W. S. Pringle for his interest and advice in the present work. This research was carried out during the tenure of the John Stothert Bye-Fellowship at Magdalene College, Cambridge, and a Senior Research Award of the Department of Scientific and Industrial Research.

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EXPLANATION OF PLATE 16

Fig. 1. Records of impulses from nerve III*b* when polarizing currents are applied between the metathoracic ganglion and this nerve. A, resting discharge; B–F, increasing negativity of the ganglion (2, 5, 10, 15 and 20 μA.); G, no current; H and I, increasing positivity of the ganglion (5 and 10 μA.). Notice the grouped discharge of small impulses in I. The time-marker indicates 20 milliseconds.

Fig. 2. Records of impulses from the giant nerve fibres of the cockroach ventral nerve cord during the polarization of the last abdominal ganglion. A, resting discharge; B–D, increasing intensities (5, 10 and 20 μA.) with the negative electrode on the ganglion; E, preparation now has a resting discharge which is decreased on the application of 5 μA. in the opposite direction. The discharge completely disappeared in this preparation at 10 μA. The time-marker indicates 10 milliseconds.

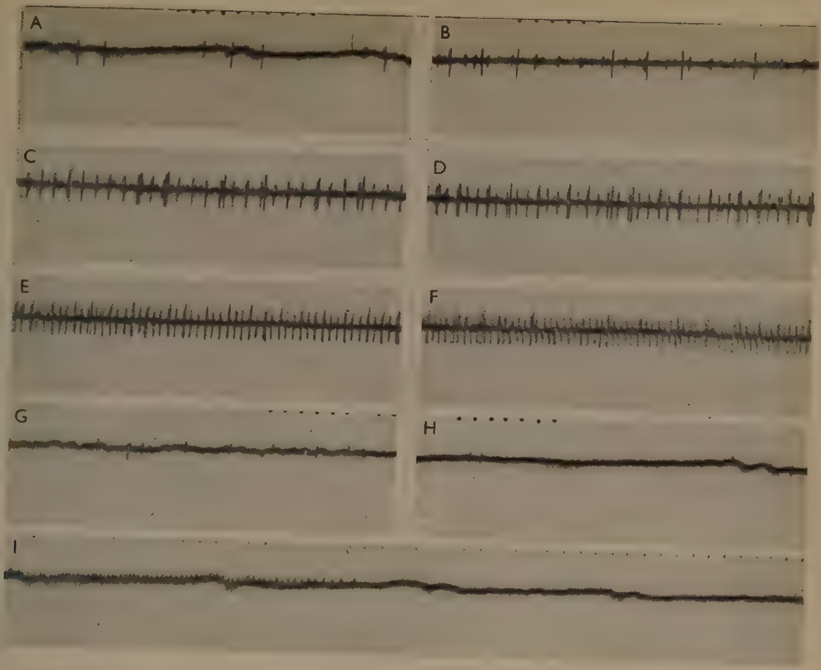

Fig. 1.

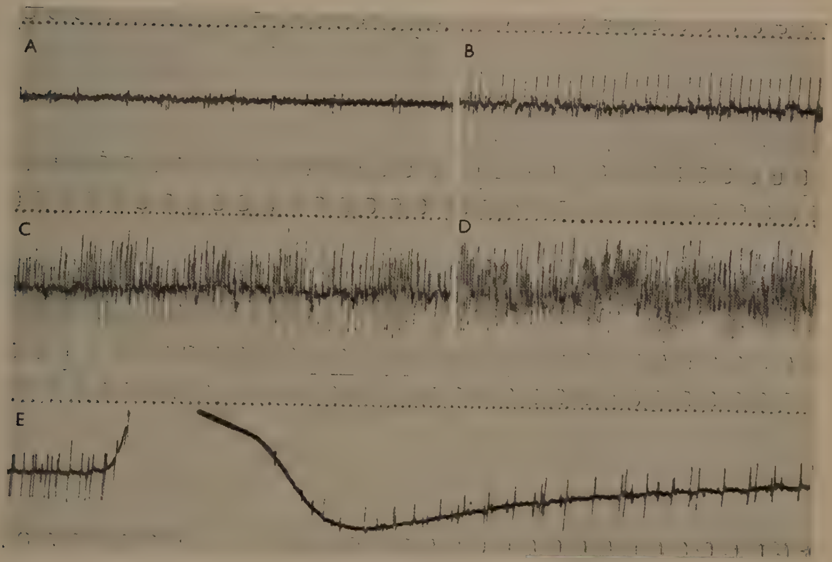

Fig. 2.

HUGHES—DIFFERENTIAL EFFECTS OF DIRECT CURRENT ON INSECT GANGLIA

THE REACTIONS OF FISH TO WATER OF LOW OXYGEN CONCENTRATION

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(*Received* 9 *December* 1951)

(With Eight Text-figures)

1. INTRODUCTION

In polluted rivers fish may encounter a great variety of toxic substances, and with the development of new industries the list of chemicals occurring as pollutants is a lengthening one. But one of the chief hazards is a lack of dissolved oxygen brought about by the decomposition of sewage or organic effluents from slaughter-houses, dairies, tanneries and paper mills. Freezing may produce a similar condition (Thompson, 1925) by cutting the water off from contact with the atmosphere. The literature on the effects of oxygen deficiency on fishes is very extensive. Gardner (1926) has a long bibliography of the older literature, and more recent work is reviewed by van Dam (1938) and Black (1951). In general previous research has consisted mainly of studies of the degree to which the oxygen consumption of fish is affected by the oxygen concentration of the water and the temperature, the comparative tolerance of different species, the effects of low oxygen concentrations on the rate and amplitude of the respiratory movements and the way the animal's power to extract oxygen from the medium is affected by the concentration of carbon dioxide. Doudoroff & Katz (1950) have recently reviewed this last aspect of the problem, and important relevant papers are those of Hart (1945), Black (1942), Fry (1947) and Fry, Black & Black (1947). There appears to have been comparatively little study of the degree to which fish can detect and avoid water of abnormally low oxygen concentration. Shelford & Allee (1913) applied their 'gradient tank' method of experiment to the problem and studied the behaviour of about sixteen American species of fish in gradients of oxygen, carbon dioxide, nitrogen and ammonia. They concluded that there appears to be little or no response to a nitrogen gradient, and that the concentration of this gas is of no importance provided that it is not present in such excess as to cause 'gas-bubble' disease; that fish react negatively to carbon dioxide and to a deficiency of oxygen, indicating their dislike by moving away, rising to the surface, gulping and 'coughing'; that fish do not react negatively to ammonia and sometimes enter concentrations which cause death. Apart from indicating that fish show a general tendency to avoid water of low oxygen concentration, the experiments of Shelford & Allee do not throw much light on the problem; they do not, for instance, indicate how far the reaction is decided by the actual concentrations of oxygen encountered or by the temperature of the water, and some of their results are rather vague; thus in one series of experiments (Shelford & Allee, 1913, p. 236, Table 9), in which eighteen fish were used, the fish spent 56% of their time in the high-oxygen part of the tank and 44% of their time in the low-oxygen part, not a difference of great significance.

The present paper records the results of about 100 experiments to test the reactions of small minnows, sticklebacks and trout to water of low oxygen concentration, using the apparatus described in earlier papers by the writer (1947, 1948). In this no gradient is produced, but two contrasted supplies of water, in and out of which the fish can swim freely, are very sharply differentiated. The general method of experiment is fully described in these papers; details relevant to the present investigation follow in the next section.

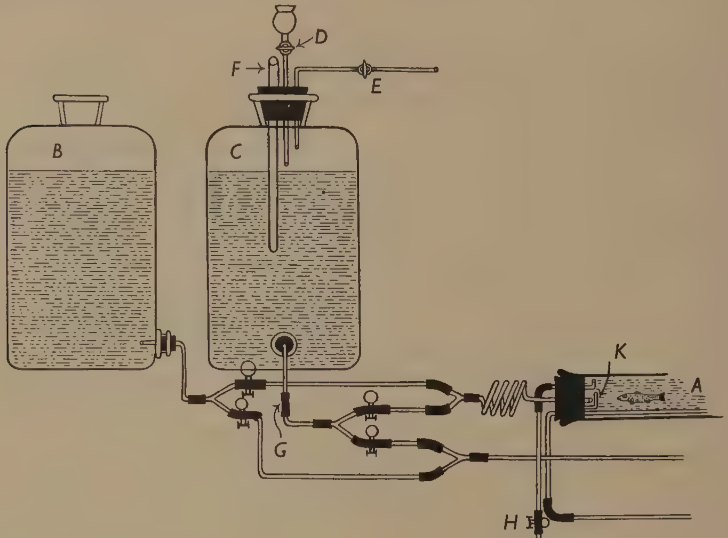


Fig. 1. Detail of apparatus. *A*, end of reaction tube; *B*, *C*, 10 l. aspirators; *D*, thistle-funnel and stopcock; *E*, stopcock connexion to filter-pump; *F*, *G*, see text; *H*, tube for expelling air from reaction tube; *K*, glass baffle in front of inlet.

2. APPARATUS AND METHOD

The apparatus used is essentially similar to that described by the writer (1948) in a paper dealing with the reactions of fish to sulphide solutions, lead nitrate and ammonia. The same arrangement has recently been used by Hodgson (1951) to study the reactions of aquatic beetles to salts and alcohols. Modifications of the original apparatus to adapt it to present requirements are shown in Fig. 1. Water of low oxygen concentration is generally prepared by boiling normal water, by subjecting it to low pressures or by passing through it a stream of nitrogen; elaborate apparatus for the preparation of large quantities of oxygen-free water has been described by Shelford (1918), and Fry (1951) has devised a fractionating apparatus to provide water of various dissolved oxygen tensions. With the apparatus used by the writer large volumes are not required, and boiling, aided by the filter pump, was found quite satisfactory. The filter pump is preferable to a vacuum pump of the 'Speedivac' oil-immersed type, as it is unaffected by the large quantities of water vapour drawn off. *A* (Fig. 1) is the reaction tube which measures internally 3·4 × 50 cm.; aspirator *B* supplies water fully saturated or slightly supersaturated with air. Aspirator *C*, of thick Pyrex glass, is detached from the apparatus and its outlet closed with a screw-clip. About 9 l. of warm or hot water is poured in, the bung is replaced, tap *D* is closed and *E* is connected to the filter pump. The pump is set working and the aspirator shaken vigorously to give a swirling motion to the water; if this is not done it boils at the top only and very little oxygen is drawn off.

The temperature of the water placed in *C* and the time the pump is operated depend on the oxygen concentration required. To produce water of very low oxygen tension it is necessary to bring it to boiling-point before pouring it into the aspirator and then boil by means of the filter pump for 15 min. or more. Boiling for a short period removes far less of the dissolved oxygen than is commonly supposed. If water 50 % saturated is required it need not be warmed at all and a few minutes' evacuation and shaking will suffice. After a certain amount of practice the temperature and evacuation time necessary to produce the required depression of the oxygen concentration can be estimated with fair accuracy. When the pump has been running for a sufficient time the tap *E* is shut and the water allowed to cool to the correct temperature with the aspirator still exhausted of air. Tube *F* permits determination of the temperature. When this is correct and equal to that of *B* pure liquid paraffin is admitted via the funnel and stopcock *D* in sufficient quantity to cover the surface of the water; air is then allowed to enter and a Winkler determination done on a sample taken by attaching a delivery tube to the rubber connexion *G* and very rapidly running about 200 ml. through a 50 ml. sampling bottle. When dealing with water of very low oxygen concentration a small sampling bottle of the type described by Powers (1918) was found useful.

The use of liquid paraffin is not always necessary. When the oxygen content of the water in the supply aspirator is not below 40 % saturation and when not more than about two-thirds of the supply is discharged during the experiment the paraffin can be omitted without serious error; this is an advantage, for cleaning the aspirator after the use of paraffin is a tedious performance. In this case, of course, the aspirator is kept exhausted of air until the experiment is begun.

When both water supplies are ready a fish is placed in the reaction tube. This is filled with water from *B*, and a little water is run in from *C* also to clear out all bubbles which are finally expelled via *H*. No bubble traps can be used for obvious reasons. The fully oxygenated supply is set running through both halves of the reaction tube; when the fish has become accustomed to its surroundings its movements are recorded for 2–3 min. and then the flow in one half of the tube is replaced by water from aspirator *C*. If the fish performs exploratory movements and visits both ends of the reaction tube at the commencement of the experiment it is preferable to start running the poorly oxygenated water through the half of the tube from which the fish is absent; if the fish is sluggish and remains in one half of the tube it is obviously necessary to admit the poorly oxygenated water on this side. When a reversal of flow is carried out normal water is passed through both halves of the tube for at least a minute before the supply from aspirator *C* is set running in the new direction. The Aberystwyth tap water is a very soft and pure water of pH normally about 6·8 and does not contain any appreciable quantity of carbon dioxide. The method used for lowering the oxygen content of the water results in the removal of some of the dissolved nitrogen. In these experiments it is assumed that fishes do not react to differences in nitrogen concentration; ideally, of course, the nitrogen should be restored so that the only difference between the two supplies of water presented to the fish is the difference in oxygen concentration.

3. EXPERIMENTS WITH STICKLEBACKS

The first experiments were carried out with sticklebacks (*Gasterosteus aculeatus* L.) at 13° C. Four typical records are given in Fig. 2, each for a single 28–32 mm. fish. The reaction to oxygen concentrations below 3 mg./l.—at this temperature about 28 % saturation—conforms to a fairly definite pattern. The fish has no immediate appreciation of the low oxygen concentration and generally swims across the boundary zone without any hesitation; occasionally it will cough, i.e. make a sudden, very jerky respiratory movement, but swim on. In a series of fifteen experiments at 13° C. with oxygen concentrations up to 3 mg./l. the fish crossed from the fully oxygenated water into the poorly oxygenated supply without hesitation about 92 times. The record for 0·8 mg. in Fig. 2, for instance, shows five such traverses between 8 and 10 min. If the fish remains in the low-oxygen zone it develops a dyspnoea, the amplitude and frequency of the respiratory movements increasing; at the same time random movement appears to be incited and the fish may struggle violently. Sooner or later this random swimming takes the animal across the boundary into the well-oxygenated water, when the stimulus to swimming disappears. Usually this happens with the greatest abruptness, so that the fish only swims about 10 cm. into the well-oxygenated water and then becomes almost motionless as its respiratory movements rapidly return to normal. Thus it appears to select the high-oxygen zone, but the basis of this selection is simply the sudden removal of the stimulus to swimming. If the poorly oxygenated supply is run through both halves of the reaction tube a stickleback will keep in continual motion up and down the tube, becoming more and more agitated; when well-aerated water is admitted the fish stops moving immediately on coming in contact with this and generally remains still until it has recovered.

Other workers have noted that a lack of oxygen stimulates swimming in fish; see, for instance, the review by Black (1951, pp. 98–9). The reaction has not been explained; it is known that the mackerel does not perform any breathing movements but keeps up a current of water through the gill chambers by swimming along with its mouth open (Scheer, 1948, p. 399), and movement through the water probably aids respiration in most fishes. Thus more rapid movement may be a natural response to oxygen deficiency.

The records in Fig. 2 are largely self-explanatory, but a description of the

0·26 mg. experiment will help to make things clear. The low-oxygen water was first admitted at 30 min. immediately after the fish had moved over to the left. Between 4 and 5 min. the right half of the tube was visited three times; the first two visits produced no great discomfort, but on the third occasion a violent dyspnoea developed with furious swimming. At 5 min. the stickleback got across the boundary and recovered. The trial at 8 min. produced no definite result, as the fish crossed the tube with a natural movement before any respiratory distress developed. At 12½ min. the 0·26 mg. water was again admitted on the right; the fish remained quite unperturbed for about 50 sec., struggled for about 40 sec., reached the well-oxygenated water, stopped and recovered, its opercular movement rate returning to normal in a little over a minute.

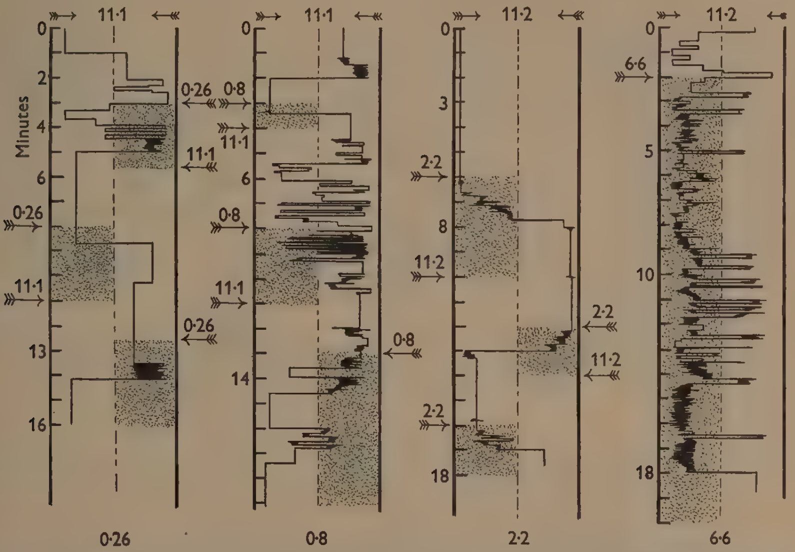


Fig. 2. The reactions of *Gasterosteus aculeatus* to water containing 0·26, 0·8, 2·2 and 6·6 mg./l. of oxygen. Temp. 13° C. Figures opposite the arrows are oxygen concentrations; thus the first experiment was begun with water containing 11·1 mg./l. flowing through both halves of the reaction tube.

When the oxygen concentration tested is 3–5 mg./l. the results are more variable. Respiratory distress, agitated swimming, apparent recognition and selection of the well-aerated water, rest and recovery generally form the behaviour pattern, the dyspnoea taking a little longer to develop. Occasionally, however, the fish does not leave the poorly oxygenated water, it does not struggle very much, and by increasing the amplitude and frequency of its breathing movements seems to succeed in obtaining enough oxygen for its needs. The fourth record in Fig. 2 is for water containing 6·6 mg./l.; in this case the stickleback seemed to have a preference for the left half of the tube, and though it made several visits to the fully aerated water it persisted in returning. In this experiment it proved possible to make some counts of the respiratory movements. These had a frequency of 96 per min. when the experiment began and had speeded up to 164 per min. when the experiment was ended at 18 min. It was then decided to make some similar observations at low oxygen concentrations; the majority of these attempts were failures, but eventually three experiments succeeded, the movements of the fish being copied together with a fair number of reasonably accurate counts of the opercular movement rate. These three records are set out in Fig. 3 and help to confirm the general interpretation of the reaction given above. In the third record in Fig. 3 the fish revisited the low-oxygen zone at 8–9 min. before it had recovered completely.

All the successful experiments carried out at 13° C. with *Gasterosteus* are summarized in Fig. 4. Here the horizontal scale gives the oxygen concentration of the 'low' zone; in every case fully oxygenated water is presented as the alternative.

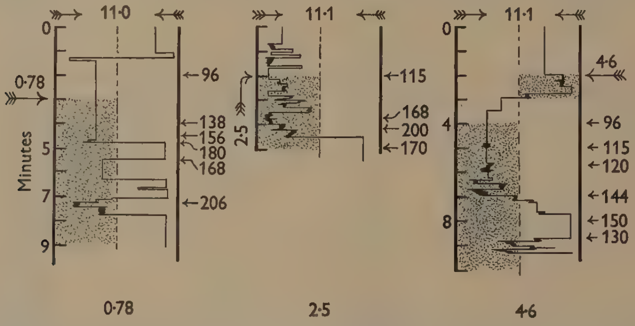


Fig. 3. Three records for *Gasterosteus* at 13° C. The figures opposite the large, tailed arrows are oxygen concentrations in mg./l.; those opposite the small arrows are respiration counts (opercular movements per minute).

The ordinates ('reaction times') are the times the fish took to leave the poorly oxygenated water. In some cases they swam into this of their own volition, in others it was admitted into the half of the reaction tube in which they had taken up a position. The reaction times are very approximate, and in some cases it was obvious that the stickleback took an inordinate time to find its way to the boundary zone. One conclusion can be drawn from Fig. 4: it is evident that as the oxygen concentration tested is decreased there is no dramatic decrease in the time taken by the fish to 'select' the well-aerated water. It should be noted that the concentration range 4·0–0·26 mg./l. represents in terms of percentage saturation a drop from 38 to 2·5%.

At low temperatures the stickleback exhibits a generally similar reaction to water of low oxygen concentration, but the whole performance is so slowed down that it can be easily watched. Several experiments were carried out at 3° C. with fish that were gradually cooled down to this temperature from 13° C. over a period of 9–10 hr. Three records are given in Fig. 5, and a description of the first experiment, with water containing 0·3 mg./l., will be sufficient. Before the low-oxygen water is

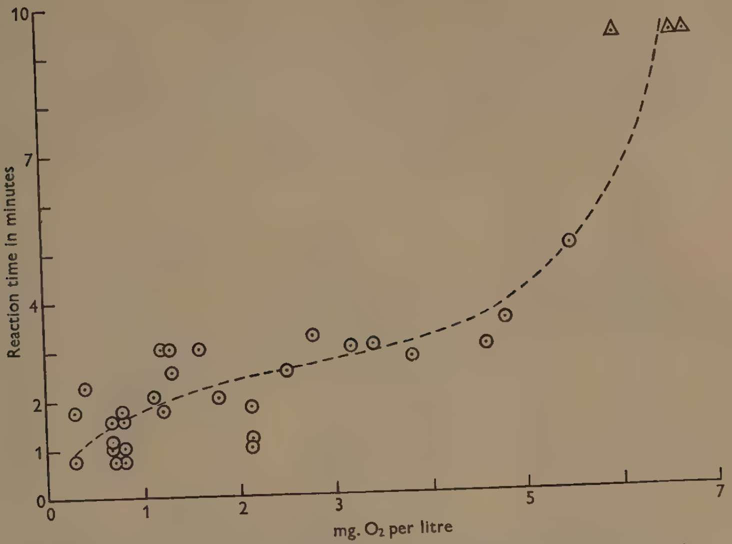


Fig. 4. Reaction times for *Gasterosteus* at 13° C. Triangles indicate concentrations at which no definite result was obtained.

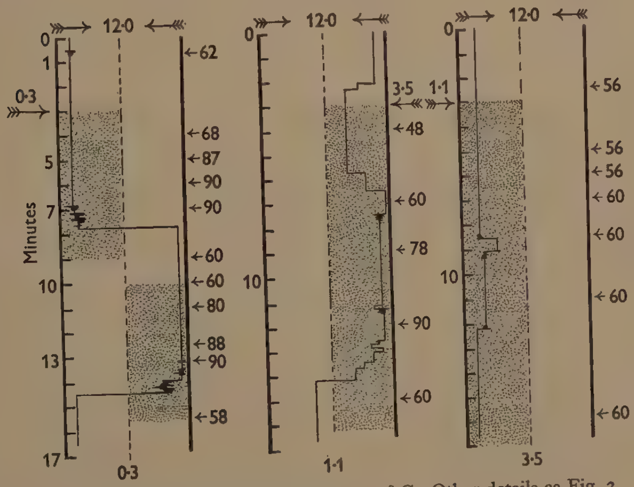


Fig. 5. Three records for *Gasterosteus* at 3° C. Other details as Fig. 3.

admitted the fish rests motionless on the bottom of the reaction tube, breathes slowly, regularly and almost imperceptibly and appears to be asleep. On admitting the 0·3 mg. water it does not move but soon the respiration rate begins to rise. At 5 min. it is 87 per min. and the opercular movements are appreciably deeper; at 6 min. they have become heaving and of great amplitude; suddenly the fish begins to swim, it crosses the boundary zone, almost immediately rests again upon the bottom of the reaction tube and once more appears to be asleep, its respiration returning to the previous frequency and amplitude. Reversal of the flows at 9–10 min. repeats the performance. In the second record in Fig. 5 it will be noted that over 9 min. go by before the boundary zone is crossed, and in the 3·5 mg. experiment the fish was motionless with no sign of respiratory distress at the end of the experiment.

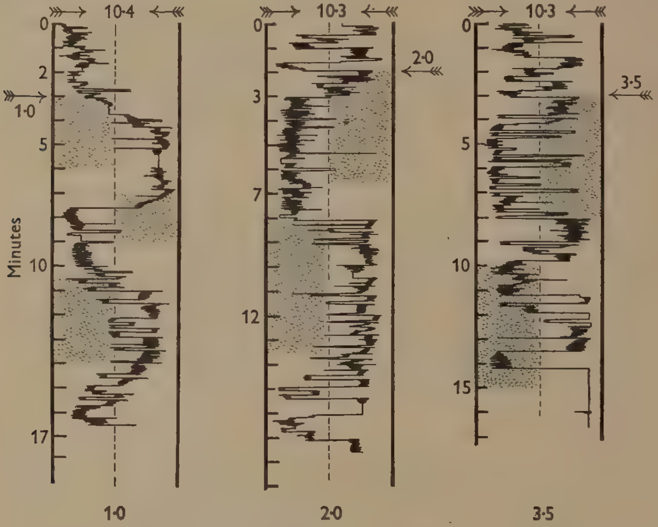
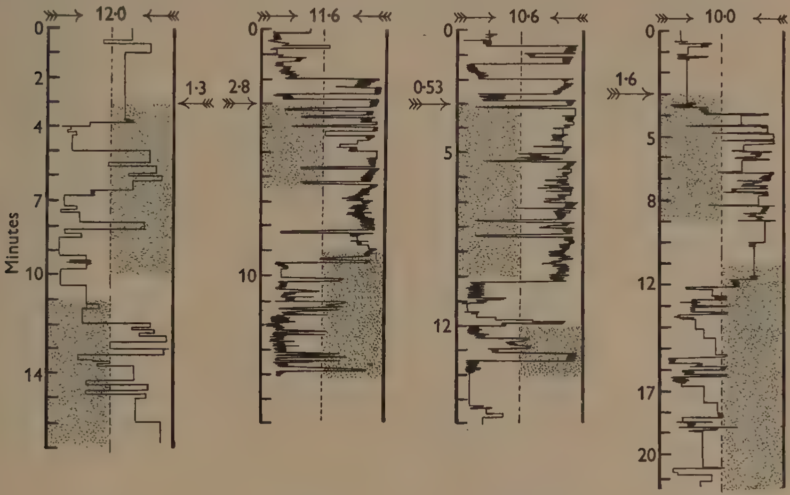


Fig. 6. Three records for *Gasterosteus* at 20° C.

At 20° C., on the other hand, when a stickleback comes into contact with water of very low oxygen concentration, respiratory difficulties seem to develop almost immediately; so rapidly that the entry of the fish is checked and it moves away, remaining in the well-aerated water (see the record for 1·0 mg./l. O_2 in Fig. 6). Thus on encountering the poorly oxygenated water the fish behaves as if it were violently irritated and retreats immediately, just as minnows appear to be violently irritated by and at once avoid a 0·03% solution of paracresol (Jones, 1951, pp. 265–6). At higher oxygen concentrations (3·5 mg., Fig. 6) the reaction is a little slower; if the fish swims at its normal speed the boundary zone is crossed, dyspnoea develops in 5–10 sec., and after some erratic movement the fish retreats. At 3·5 mg. the reaction appears to be a much speeded-up version of that seen at lower temperatures; whether the animal's behaviour at 20° C. and very low oxygen concentrations is a further speeding-up of the same sequence of events is not clear. It is impossible to avoid the conclusion, however, that the essential feature of the reaction is the rate of development of dyspnoea; it may be that at any particular low oxygen concentration there is a critical temperature at which respiratory distress is developed so quickly that a fish swimming at the normal rate is turned back at once. The reaction fails occasionally when the fish crosses the boundary zone with a wild rush (see Fig. 6, 2·0 mg., 5–6 min.).



1·3 mg.
3° C.

2·8 mg.
12·5° C.

0·53 mg.
18° C.

1·6 mg.
24° C.

Fig. 7. The reactions of *Phoxinus phoxinus* to water containing 1·3 mg. oxygen per litre at 3° C.; 2·8 mg. at 12·5° C.; 0·53 mg. at 18° C.; and 1·6 mg. at 24° C.

It will be noted in the 1·0 mg. experiment recorded in Fig. 6 that the responses at 4–6, 8–9 and 12–14 min. are much more prompt than those displayed on the admission of the poorly oxygenated water at 3, 7 and 10½ min., but when it is remembered that the poorly oxygenated water takes some 30 sec. to replace the previous supply the explanation for this is obvious.

4. EXPERIMENTS WITH MINNOWS, *PHOXINUS PHOXINUS* (L.)

With minnows twenty-four experiments were carried out at temperatures from 3 to 24° C. and oxygen concentrations from 0·27 to 6·0 mg./l.; four representative results are given in Fig. 7. The behaviour of the fish is essentially similar to that observed in the case of *Gasterosteus* but is less dependent on the temperature, so that the reaction at low temperatures is less delayed. In the 3° C. 1·3 mg. experiment the first reaction was rather indefinite, but after 7 min. the fish responded comparatively promptly; it would swim across the boundary zone, develop gulping respiratory movements of great amplitude in 20 sec. or less and return to the well-oxygenated water. At the same temperature 3·0 mg./l. of oxygen produced a fairly good response in about 4 min., but water containing 4·6 mg./l. produced neither dyspnoea nor movement in 15 min. In the 2·8 mg. 12·5° C. experiment the fish swam about fairly quickly, would penetrate some distance into the low-oxygen zone, hesitate 3–5 sec., begin gulping wildly and retreat. At 0·53 mg. and 18° C. the minnow was very active, would rush into the poorly oxygenated water, become very distressed almost immediately and rush out; at 13 min. the fish swam deep into the right half of the tube and began violent struggles which did not get it to the boundary zone until about 25 sec. had elapsed, by which time it had lost its sense of balance. Swimming on one side it reached the well-oxygenated water and recovered in about 2 min.

The 24° C. experiment was one carried out rather later in the spring (June) when the minnows had become more acclimatized to high temperatures. In this trial the fish did not swim so wildly and generally would not penetrate more than 2–3 cm. into the low-oxygen zone, developing respiratory difficulties almost immediately. At this temperature lack of oxygen very quickly produces in minnows an extremely high opercular movement rate exceeding 270 per min., and an avoiding reaction is promptly developed to water containing up to 4·0 mg./l.

The minnows seemed to be much more adept than the sticklebacks at finding their way about in the reaction tube, and when stimulated to swim by respiratory difficulties would very often move at once in the right direction. This was particularly evident in the case of the fish used in the 1·3 mg. experiment recorded in Fig. 7. Not all the fish behaved in this way; some, when stimulated by oxygen lack, began what appeared to be a random movement.

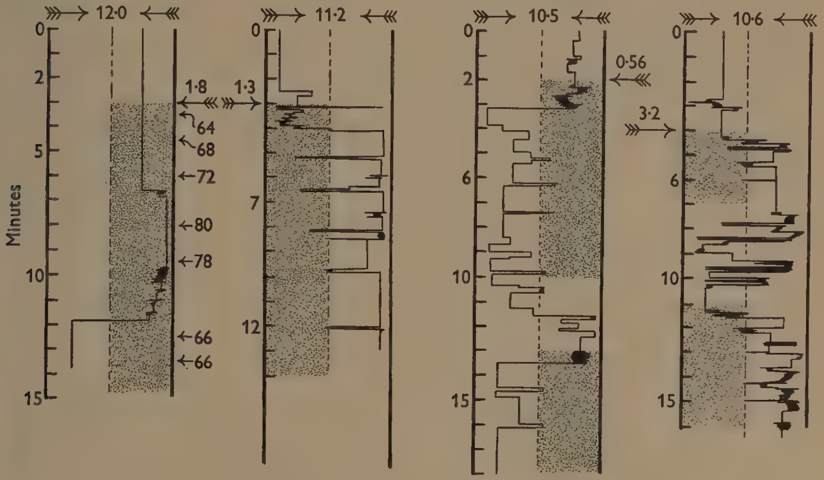
5. EXPERIMENTS WITH TROUT FRY, *SALMO TRUTTA* L.

A number of trials were made with 30–32 mm. trout fry, and four typical results are given in Fig. 8. At 3° C. the response takes some time to develop; at first the fish breathes very quietly and regularly about 64 times per min., and resting on the bottom of the tube appears to be asleep. When the water of low oxygen concentration is run in the respiratory movements gradually quicken and attain a surprising amplitude; the fish, still resting on the bottom, raises itself on its pectoral fins with its body arched backwards in a curious posture resembling opisthotonos. Eventually it begins to swim in an uneasy, aimless fashion, and on crossing into the well-oxygenated water immediately 'falls asleep' again. At 13° C. the response is much more prompt; if the fish swim across the boundary zone they develop a marked dyspnoea in 5–7 sec. and return to the well-oxygenated water.

Some of the experiments at 13° C. were not very satisfactory, for the trout, which were obtained from a hatchery, appeared to be very tame and tended to remain motionless for long periods, making few voluntary excursions to the poorly oxygenated water. At 20° C. the fish were more active, and the response to a concentration of 0·56 mg./l. was so rapid that the behaviour of the fish appeared to be an immediate recognition and rejection of the poorly oxygenated water, for on swimming into it the trout would stagger, gulp violently and retreat in less than 5 sec. A 3·2 mg. experiment at this temperature also resulted in a very good avoiding reaction.

6. DISCUSSION

In a general discussion of their results Shelford & Allee (1913, p. 261) note 'that fishes are able to react to stimuli by simple turnings back, and that as a rule, they remain longer in water which does not clearly influence the details of their activities'. In the course of subsequent work to test the reactions of fishes to various polluting chemicals Shelford (1917) obtained results suggesting that fish react 'positively' to a great number of polluting chemicals, swimming into the solution as if attracted by it, remaining in it and even avoiding pure water. On the basis of these experiments the assumption appears to have been made that while fish have no instinctive, protective reaction to poisonous, unfamiliar substances, they do have an instinctive capacity for actively avoiding water of abnormally low oxygen concentration, a condition they may be accustomed to meeting in their normal environment. Thus Ellis (1937, p. 372), after an extensive survey of fresh waters in America involving 5809 oxygen determinations at 982 stations, concluded that 'these data collected from localities where the fish had had opportunity to choose for themselves point very strongly to 5 p.p.m. as the lower limit of dissolved oxygen, if the complex is to maintain a desirable fish faunae under natural river conditions'. Eriksen & Townsend (1940, p. 84) in a study of pollution by waste sulphite liquors refer to Ellis's work stating that it supplies evidence 'that salmon and trout—"representatives



1·8 mg. 3° C.

1·3 mg. 13° C.

0·56 mg. 20° C.

3·2 mg. 20° C.

Fig. 8. The reactions of *Salmo trutta* (fry) to water containing 1·8 mg. oxygen per litre at 3° C.; 1·3 mg. at 13° C.; 0·56 and 3·2 mg. at 20° C.

of the fine fish group"—either select water having dissolved oxygen content of 4 p.p.m. or more, or such fish find it impossible to establish important populations in waters having less than 4 p.p.m.' Thompson (1925, p. 429), discussing the reactions of fish to oxygen deficiencies in the Illinois river, attributes to fishes an ability to avoid concentrations that would be fatal to them. The writer's experiments described in this paper appear to indicate that fish do not have any instinctive ability to recognize immediately and avoid water of abnormally low oxygen content, that they swim into it with little or no hesitation when the oxygen concentration and temperature do not provoke immediate and acute respiratory distress, and if they eventually avoid it the basis of the reaction would appear to be the active, random swimming and struggling that appears to be incited by dyspnoea. A full discussion of the application of the results to problems of river pollution would be beyond the scope of this paper, but it may be pointed out that in further investigations the temperature factor must be regarded as one of the greatest importance. That the oxygen demands of fishes vary greatly with the temperature is well known; van Dam (1938, p. 71), for instance, found that a rise of about 8° C. caused the oxygen intake of a rainbow trout to increase from 42·5 to 98·0 c.c./hr., equivalent to a Q_{10} of about 2·7. More significant than the increase in oxygen demand is the increase in the rate at which dyspnoea develops; this appears to be the essential factor deciding whether fish will enter water of a different oxygen concentration. When we consider, for example, that at 3° C. water containing about 0·5 mg. O_2/l. may be tolerated by *Gasterosteus* for over 5 min., whereas the same concentration at 20° C. brings on a severe dyspnoea in less than 5 sec., the importance of the temperature factor is clear. Under natural conditions, however, we have to take into consideration the degree to which polluted and unpolluted waters may mix to form gradients, the presence in polluted water of specific toxic substances, such as the sulphides produced by the decomposition of sewage (Longwell & Pentelow, 1935), the concentration of carbon dioxide and the degree to which a more rapid appreciation of unfavourable conditions at high temperatures is counterbalanced by greater oxygen demands.

SUMMARY

1. This paper presents the results of about 100 experiments to test the reactions of small fish to water of low oxygen concentration, using an apparatus similar to that previously employed by the writer to study the reactions of fish to toxic solutions.

2. At 13° C. the stickleback (*Gasterosteus aculeatus*) has no immediate appreciation of water of low oxygen concentration and will swim into it with no hesitation. If the fish remains in the poorly oxygenated water respiratory distress develops, accompanied by uneasiness and random movement. When this random movement takes the animal back into the well-oxygenated water the stimulus to swimming vanishes, the fish stops moving and quickly recovers. Over a wide concentration range at 13° C. the reaction takes 1–3 min. At 3° C. the reaction is very slow, taking 5–6 min. even when the water contains only 0·3 mg./l. of oxygen. At 20° C. and

oxygen concentrations below 2 mg./l. the response is very prompt, respiratory distress developing so quickly that the fish usually will not swim into the poorly oxygenated water, but turns away from it or swims backwards; it may make repeated attempts to enter the low-oxygen zone, retreating each time as if violently irritated.

3. Experiments with minnows (*Phoxinus phoxinus*) and trout fry (*Salmo trutta*) gave generally similar results. Here again the essential factor appears to be the speed with which dyspnoea develops.

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THE METABOLISM OF THE SEA-URCHIN EGG

OXIDATION OF CARBOHYDRATE

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(*Received* 13 *December* 1951)

(With Five Text-figures)

INTRODUCTION

The evidence that eggs of *Echinus esculentus* contain the enzymes of a glycolytic system capable of functioning under anaerobic conditions has been given in an earlier paper (Cleland & Rothschild, 1952).

A purely oxidative pathway for hexose breakdown has been described in various mammalian tissues, in yeast and in certain bacteria, by Dickens (1936, 1938*a*, *b*), Dickens & Glock (1951), Cohen & Scott (1950) and Cohen (1951). Cohen (1950) has also found this system in a number of algae and in the spermatozoa of *Chaetopterus*. This pathway, which may be termed the hexose monophosphate shunt, diverges from the normal glycolytic pathway at glucose-6-phosphate. Oxidation of this substance yields phosphogluconate which, by primary oxidation and subsequent decarboxylation, yields ribose-5-phosphate. Ribose-5-phosphate appears to have two possible fates; further oxidation to largely unknown intermediates (Dickens, 1938*a*), or breakdown by aldolase action to phosphoglyceraldehyde (Racker, 1948) which re-enters the glycolytic pathway. All the oxidative steps in this chain of reactions are catalysed by triphosphopyridine nucleotide (TPN).

It has been suggested, most recently by Lindberg & Ernster (1948), that the hexose monophosphate shunt is the principal mechanism of carbohydrate breakdown in the sea-urchin egg. The evidence for and against this theory has been reviewed by Rothschild (1951), the most important points being: (*a*) homogenates actively metabolize phosphogluconate; (*b*) low concentrations of iodoacetate do not inhibit the endogenous O_2 uptake of homogenates; (*c*) the addition of hexose monophosphate causes a greater stimulation of O_2 uptake than hexose diphosphate; (*d*) significant amounts of the phosphorylated intermediates of glycolysis could not be identified by Ba fractionation of acid egg extracts.

The experiments of Lindberg & Ernster (1948) were all performed in the presence of fluoride, final concentration M/10. This would almost certainly abolish pyruvate production, so that any increase in O_2 uptake observed on hexose addition could only be due to the hexose monophosphate shunt or to the oxidation of phosphoglyceraldehyde formed by a glycolytic mechanism.

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The purpose of the experiments described in this paper was to find out whether the glycolytic system, which functions under anaerobic conditions, also functions aerobically, or whether it is replaced by the hexose monophosphate shunt. The experiments show that the hexose monophosphate shunt is unlikely to play any great part in the oxidative metabolism of carbohydrate, and that the normal glycolytic and tricarboxylic acid cycles can account for most of the observations.

MATERIAL AND METHODS

The methods of preparing egg suspensions of *Echinus esculentus* were described in our previous paper (Cleland & Rothschild, 1952). Homogenates were made by suspending one volume of packed, jelly-free eggs in 1–1·3 volumes of M/50 phosphate buffer, pH 7·5. Complete cytolysis occurred.

Elaborate precautions to exclude Ca^{++} from the homogenates (Hultin, 1950) seemed unnecessary with the material used in these experiments. The rapidly declining O_2 uptake found by Hultin in Ca^{++}-contaminated homogenates was not observed, the O_2 uptake rates being almost linear for prolonged periods. The addition of Ca^{++} to the homogenates did, however, produce a similar effect to that described by Hultin (Fig. 5).

Reagents, phosphorylated intermediates, and analytical methods were described in our previous paper. Crystalline Na pyruvate was prepared by the method of Robertson (1942). O_2 uptake was measured in Warburg manometers with a flask capacity of 14–18 ml.; the temperature was 20° C. and the shaking rate 90 c.p.m. with a 4 cm. stroke. Unless otherwise stated, the flasks contained: phosphate buffer, pH 7·5, final concentration M/20, cytochrome *c* (60 % purity, L. Light & Co.), final concentration $1{\cdot}5 \times 10^{-5}$M, and 1 ml. of homogenate, in a final volume of 2 ml.

RESULTS

Homogenate medium. The endogenous O_2 uptake of homogenates, prepared as described above, was compared with that of homogenates in a mixture of 0·3 M-KCl and phosphate buffer, pH 7·5, final concentration M/50. No differences were found between the two, but the former was used because it oxidized added pyruvate and succinate more actively than the latter.

Effect of cytochrome c. When cytochrome *c* was added to homogenates there was a marked increase in O_2 uptake and less tendency for the rate to decline with time (Fig. 1).

Effect of inhibitors. Fluoride, iodoacetate, phenylmercuric nitrate, malonate and fluoracetate inhibited O_2 uptake (Table 1).

Effect of tricarboxylic acid cycle intermediates. The O_2 uptake of homogenates was significantly increased on addition of citrate, α-ketoglutarate, succinate, fumarate, malate and glutamate (Table 2). The stimulation after addition of pyruvate was somewhat variable; this is discussed later.

Pyruvate metabolism. Those homogenates whose O_2 uptake was relatively unaffected by the addition of pyruvate (5–15 % stimulation) were able to utilize the added pyruvate. Furthermore, pyruvate accumulated in the presence of HCN

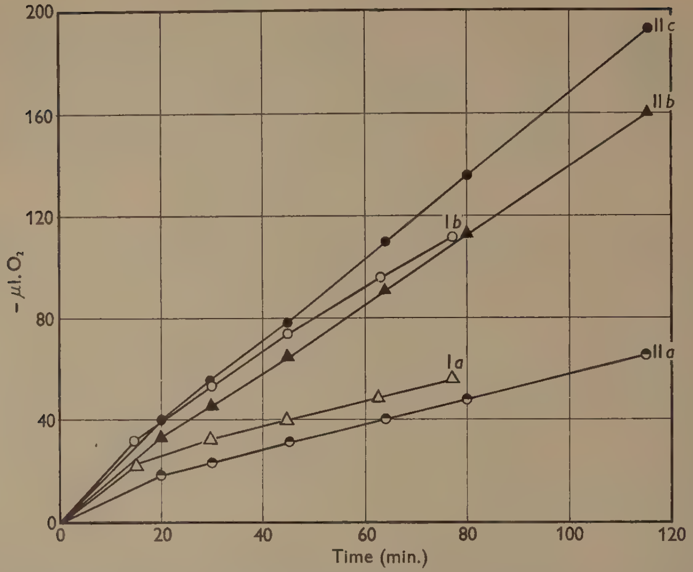

Fig. 1. Effect of cytochrome *c* on endogenous O_2 uptake of egg homogenates. I*a*, control; I*b*, O_2 uptake in presence of $1{\cdot}5 \times 10^{-5}$ M cytochrome *c*; II*a*, control; II*b* and *c*, O_2 uptake in presence of $7{\cdot}5 \times 10^{-6}$ M and 3×10^{-5} M cytochrome *c*.

Table 1. *Effect of inhibitors on the endogenous* O_2 *uptake of egg homogenates*

Duration of experiment, 90 min.

Inhibitor	Conc. (M)	% inhibition	No. of experiments
Iodoacetate	10^{-2}	46	1
	10^{-3}	22	2
Fluoride	10^{-2}	37	4
	10^{-3}	16	4
Phenyl mercuric nitrate	10^{-3}	63	2
Malonate	10^{-2}	44	2
Fluoracetate	10^{-2}	23	2

Table 2. *Effect of tricarboxylic acid cycle intermediates on the* O_2 *uptake of egg homogenates*

Duration of experiment, 90 min.

Substrate	% control	No. of experiments
Citrate, 10^{-2}M	135	3
α-ketoglutarate, 10^{-2}M	145	2
Succinate, 10^{-2}M	142	3
Fumarate, 10^{-2}M	115	2
Malate, 10^{-2}M	121	4
Glutamate, 10^{-2}M	110	2
Pyruvate, 10^{-2}M	120	7

(Table 3), suggesting that it was arising during endogenous metabolism but was being removed immediately by oxidation.

Table 3. *Pyruvate utilization by egg homogenates*

Duration of experiment, 60 min.

Exp. no.	System	µl. O_2	Terminal change	
			µg. pyruvate	µg. lactate
1	Homogenate	72	+ 2	0
2	(1)+pyruvate, 110 µg./flask	81	−100	+12
3	(1)+fumarate, 10^{-2} M	83	—	—
4	(3)+pyruvate, 110 µg./flask	90	−105	+12
5	(1)+cyanide, 10^{-3} M	10	+ 20	+60

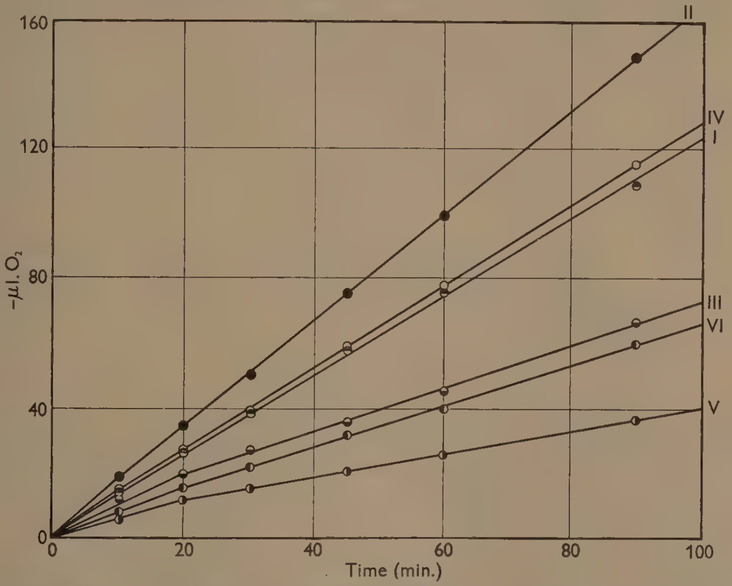

Fig. 2. Effect of fluoride and dilution on pyruvate metabolism of homogenates in phosphate buffer + cytochrome *c*. I, control; II, final pyruvate concentration, 0·01 M; III, final fluoride concentration, 0·005 M; IV, pyruvate, 0·01 M and fluoride, 0·005 M; V, control, but with enzyme concentration reduced to half that in I; VI, the same as V but with pyruvate, 0·01 M.

In preparations showing a moderate initial stimulation of O_2 uptake (20–40 %) on addition of pyruvate, the effect may be increased by reducing the rate of endogenous pyruvate production. Fluoride at a concentration of M/200 inhibits glycolysis by about 85 % (Cleland & Rothschild, 1952). When pyruvate is added to a fluoride-poisoned system, the observed stimulation is more than double that in controls (Fig. 2). In mammalian tissues glycolytic enzymes are to a large extent soluble, while the enzymes of the tricarboxylic acid cycle are usually bound to cell granules. This distribution of enzymes probably occurs in invertebrate eggs as well (Cleland, 1951). This being so, dilution of a homogenate would reduce the glycolytic rate more than the oxidative rate and thus be similar to fluoride in its effect on pyruvate metabolism. The experiment in Fig. 2 confirms this.

When the initial response to pyruvate addition is weak (5–15 % stimulation of O_2 uptake), the above procedures have little effect. This suggests that the poor response of these preparations to pyruvate addition was not due to a high endogenous pyruvate production but to some interference with the system responsible for pyruvate oxidation.

Pyruvate oxidation can proceed to completion in active preparations (i.e. $2\frac{1}{2}$ moles O_2/mole utilized pyruvate), while in the less active ones, the extra oxygen consumed varied between the above extreme and one equivalent to $\frac{1}{2}$ mole O_2/mole utilized pyruvate (Fig. 3). The addition of fumarate did not increase the efficiency of utilization of pyruvate in the latter type of preparation (Table 3), showing that the inefficiency was not due to lack of oxalacetate.

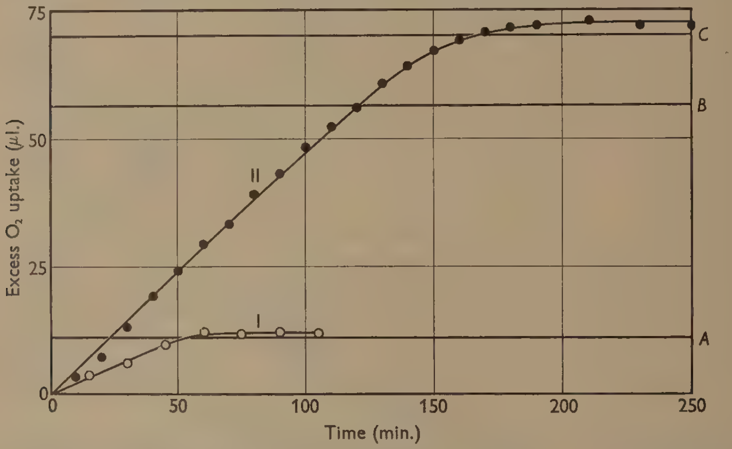

Fig. 3. Oxidation of added pyruvate by egg homogenates. I, oxidation of 110 μg. Na pyruvate by an inactive preparation; II, oxidation of 110 μg. Na pyruvate by an active preparation. The horizontal lines *A* and *B* show the theoretical excess O_2 uptake for $\frac{1}{2}$ O_2/mole pyruvate and $2\frac{1}{2}$ O_2/mole pyruvate, the latter being complete combustion. Line *C* shows the theoretical O_2 uptake for complete combustion of pyruvate + lactate. In curve II, 20 μg. more lactic acid disappeared than in the control, in addition to the complete disappearance of the added pyruvate.

Effect of carbohydrates. Glycogen, glucose and fructose increased the O_2 uptake of homogenates (Table 4).

The endogenous O_2 uptake of homogenates and the oxidation of fructose were stimulated by diphosphopyridine nucleotide (DPN) and adenosinetriphosphate (ATP), but were inhibited by iodoacetate (IAA) and fluoride (Tables 5 and 6).

Effect of phosphorylated intermediates. The addition of various phosphorylated intermediates of glycolysis and of phosphogluconate increased the O_2 uptake of homogenates. The results of a number of experiments are given in Table 7 while the rate curves of one experiment are shown in Fig. 4. The hexose diphosphate (HDP) was originally a Ca salt which was later converted to a Ba salt. The possibility that Ca contributed to the HDP effect had therefore to be considered (Hultin, 1950). When Ca was added to homogenates, however, there was a marked and sudden increase in the rate of O_2 uptake which soon declined, so that after 2–4 hr. the total O_2 consumed in the presence of Ca was less than that of the controls. When HDP was added, the increased rate of O_2 uptake was maintained for long periods (Fig. 5).

Table 4. *Effect of carbohydrates on O_2 uptake of egg homogenates*

Duration of experiment, 75 min.

Addition	μl. O_2	%
Nil	110	100
Glycogen, 10^{-2}M*	121	110
Glucose, 10^{-2}M	138	125
Fructose, 10^{-2}M	153	139

* Glucose equivalent.

Table 5. *Effect of cofactor additions on endogenous O_2 uptake and the oxidation of fructose by egg homogenates*

Duration of experiment, 120 min.

Exp. no.	System	μl. O_2
1	Homogenate	122
2	(1)+DPN, 200 μg.	157
3	(1)+ATP, 1 mg.	139
4	(3)+DPN, 200 μg.	179
5	(1)+fructose, 10^{-2}M	184
6	(5)+DPN, 200 μg.	262
7	(6)+ATP, 1 mg.	272
8	(4)+IAA, 5×10^{-3}M	115
9	(7)+IAA, 5×10^{-3}M	135

Table 6. *Effect of inhibitors on fructose oxidation by egg homogenates*

Duration of experiment, 120 min.

Exp. no.	System	μl. O_2	Excess O_2	% inhibition
1	Homogenate	183	—	—
2	(1)+fructose, 10^{-2}M	234	51	—
3	(1)+fluoride, 5×10^{-3}M	141	—	23
4	(3)+fructose, 10^{-2}M	154	13	75
5	(1)+iodoacetate, 5×10^{-3}M	125	—	32
6	(5)+fructose, 10^{-2}M	140	15	71

Table 7. *Effect of phosphorylated intermediates on O_2 uptake of homogenates*

Substrate	% control	No. of experiments
Glucose-1-phosphate, 5×10^{-3}M	115	2
Glucose-6-phosphate, 5×10^{-3}M	136	5
Phosphogluconate, 5×10^{-3}M	122	8
Fructose-6-phosphate, 5×10^{-3}M	130	3
Fructose-1:6-diphosphate, 5×10^{-3}M	146	7

Phosphogluconate oxidation. The oxidation of phosphogluconate differed in several respects from that of glucose-6-phosphate. The oxidation of the latter was enhanced by DPN which had no effect on the former. Some pyruvate and lactate accumulation occurred during the oxidation of glucose-6-phosphate, but not in the case of phosphogluconate (Table 8). The aerobic accumulation of pyruvate and lactate was greater when the substrate was HDP than when it was glucose-6-phosphate; a similar difference between these two substrates was noted in anaerobic experiments (Cleland & Rothschild, 1952).

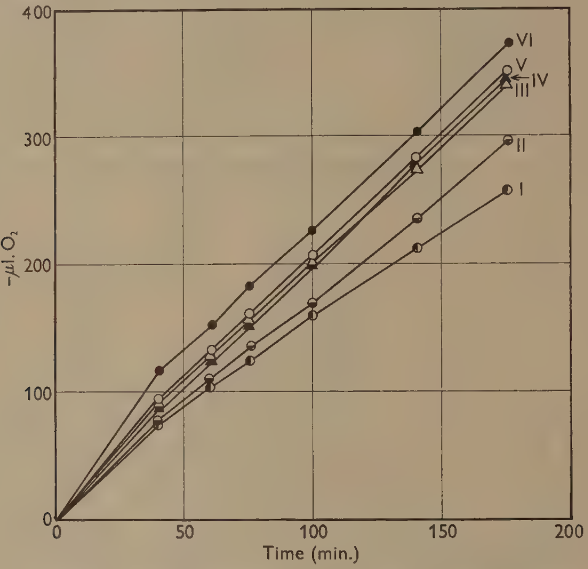

Fig. 4. Effect of phosphorylated intermediates on O_2 uptake of egg homogenates. I, control; II–VI, effect, respectively, of adding glucose-1-phosphate, glucose-6-phosphate, phosphogluconate, fructose-6-phosphate, and hexose diphosphate; final concentrations in each case, 5×10^{-3}M.

DISCUSSION

Cytochrome system. The evidence for the occurrence of an 'ordinary' cytochrome system in the sea-urchin egg has been discussed by Rothschild (1949, 1951) and Borei (1951). Although cytochrome *c* has never been directly demonstrated in these eggs, there is a case for there being adequate amounts to support the greater part of the respiration of intact eggs. The addition of cytochrome *c* to homogenates stimulates the endogenous O_2 uptake of homogenates, a fact also noted by Crane & Keltch (1949), Ycas (1950) and Keltch, Strittmatter, Walters & Clowes (1950).

Inhibitors. The experiments with inhibitors are consistent with the operation of a glycolytic and tricarboxylic acid cycle. The inhibitory action of malonate is somewhat greater than has so far been found in invertebrate tissues, though Ycas (1950) found an inhibition of the same order in egg homogenates of *Strongylocentrotus* *purpuratus*. In other invertebrate tissues such as oyster eggs (Cleland, 1950), earthworm preparations (O'Brien, personal communication) and oyster muscle (Humphrey, 1947), malonate is an inefficient inhibitor. Fluoracetate is a powerful inhibitor of acetate metabolism in animal tissues (Bartlett & Barron, 1947). The small effect of this compound on sea-urchin eggs is interpreted to mean that acetate metabolism plays only a small part in the overall oxidative metabolism of this system. The inhibition of the endogenous O_2 uptake of homogenates by iodoacetate is at variance with the results of Lindberg & Ernster (1948), who reported that a concentration of 0·005 M of this substance had little effect on their

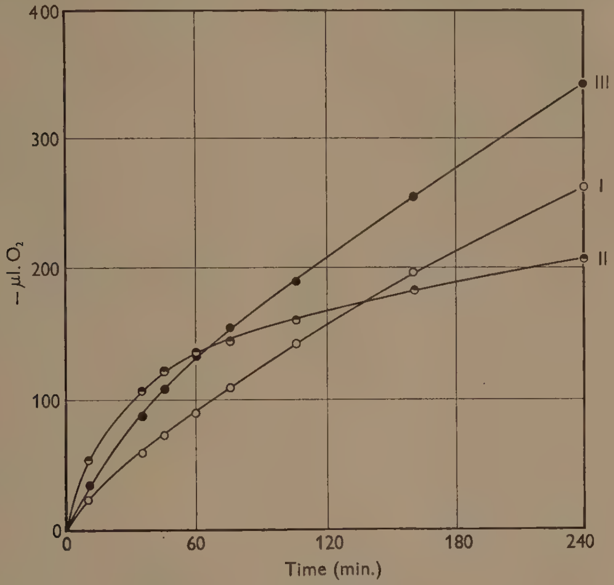

Fig. 5. Effect of Ca^{++} and hexose diphosphate on O_2 uptake. I, control; II, concentration of added Ca^{++}, 0·005 M; III, concentration of added hexose diphosphate, 0·005 M.

Table 8. *Effect of cofactor addition*

Duration of experiment, 160 min.

Exp. no.	System	µl. O_2	µl. O_2 excess	Terminal change	
				µg. lactate	µg. pyruvate
1	Homogenate	135	—	—	—
2	(1) + DPN, 100 µg./ml.	216	—	+ 1	+ 5
3	(1) + phosphogluconate, 5×10^{-3} M	164	29	0	0
4	(2) + phosphogluconate, 5×10^{-3} M	248	32	+ 2	+ 6
5	(1) + glucose-6-phosphate, 5×10^{-3} M	168	33	+ 2	+ 5
6	(2) + glucose-6-phosphate, 5×10^{-3} M	264	48	+ 10	+ 40

preparations, which were obtained from *Strongylocentrotus droebachiensis*. As Hultin (1950) found that 5×10^{-3}M-iodoacetate inhibited the O_2 uptake of egg homogenates of *Paracentrotus lividus*, species differences may explain these apparent discrepancies.

Tricarboxylic acid cycle. The evidence for the operation of a tricarboxylic acid cycle in the eggs of *Echinus esculentus* is: (*a*) the inhibition of endogenous O_2 uptake of homogenates by malonate; (*b*) the stimulation of O_2 uptake on addition of cycle intermediates; (*c*) the complete oxidation of pyruvate. The latter is the most cogent evidence because the tricarboxylic acid cycle is the only mechanism known in animal tissues which will combust pyruvate to CO_2 and H_2O.

The respiratory stimulation caused by the addition of tricarboxylic acid cycle intermediates is generally smaller than that found in the oyster egg (Cleland, 1950) but is of the same order as that in the eggs of *Arbacia punctulata* (Crane & Keltch, 1949; Keltch *et al.* 1950). The effect is somewhat smaller in the eggs of *Strongylocentrotus purpuratus* (Ycas, 1950). Lindberg (1943) has described a stimulating effect of malate and fumarate in egg homogenates of *Echinocardium*.

Preparations differed a good deal in the extent to which the addition of pyruvate stimulated O_2 uptake and in the efficiency with which added pyruvate was oxidized. In the latter case two extremes were found: first, the uptake of $\frac{1}{2}$ mole O_2 and secondly, the uptake of $2\frac{1}{2}$ moles O_2 per mole, of utilized pyruvate. These two extremes, and the intermediate values, are consistent with the reactions of pyruvate known to occur in other organisms. According to Ochoa (1951), pyruvate reacts indirectly with coenzyme A to yield acetyl coenzyme A, this reaction resulting in the absorption of $\frac{1}{2}$ mole O_2/mole pyruvate. Acetyl coenzyme A has three possible fates: (*a*) combination with phosphate to yield acetyl phosphate; (*b*) combination with acetyl phosphate to give acetoacetate; (*c*) combination with oxalacetate (catalysed by the 'condensing enzyme') to give citrate which may then enter the tricarboxylic acid cycle. The observed uptake of $\frac{1}{2}$ mole of O_2/mole of pyruvate would be consistent with reactions (*a*) or (*b*); the uptake of $2\frac{1}{2}$ moles of O_2 would be consistent with reaction (*c*). Intermediate values would result from varying proportions of (*c*) and (*a*) or (*b*).

If this interpretation is correct for these homogenates, the activity of the condensing enzyme would appear to limit reaction (*c*). This follows from the fact that an increase in oxalacetate concentration did not increase the efficiency of pyruvate oxidation and that citrate was actively oxidized in all experiments in which it was added.

The following observations leave little doubt that pyruvate was being produced and oxidized during the endogenous metabolism of the egg homogenates: (*a*) when pyruvate production was reduced by fluoride or dilution, added pyruvate gave a greater stimulation of O_2 uptake than in the controls; (*b*) when a carbohydrate substrate was added accumulation of pyruvate and lactate occurred and the oxygen uptake was increased. In this case the rate of production of pyruvate exceeded its rate of oxidative removal; (*c*) when oxidative metabolism was blocked by cyanide an accumulation of pyruvate and lactate occurred.

An estimate of the pyruvate metabolism in homogenates can be made from the experiment in Fig. 2. The time course of oxidation in the fluoride-poisoned homogenate is not linear, which is almost certainly due to the oxidation of the lactate and pyruvate present in the homogenate at the beginning of the experimental period. Taking the period 30–90 min., during which all the rates are linear, it is found that the inhibition by fluoride of the oxidation mechanism itself is 22 %. If this were the only inhibition, the O_2 uptake of the fluoride-poisoned homogenate would be 57 μl., whereas the observed value is 37 μl. The O_2 uptake from pyruvate oxidation would thus be 20 μl., about 85 % of the true value (since glycolysis is only 85 % inhibited at that fluoride concentration). The true value is therefore 23 μl. which means that oxidation of pyruvate accounts for some 32 % of the total oxygen uptake of the homogenate. The 23 μl. O_2 would be taken up during the complete oxidation of 40 μg. pyruvate. The experiment in Table 3 and those in our previous paper show that this amount of pyruvate can be produced.

Hexose metabolism. There is a comparable increase in O_2 uptake (this paper) and in lactic acid production (Cleland & Rothschild, 1952) after addition of various carbohydrates to egg homogenates. This parallel was confirmed by experiments in which we examined the effects of DPN and ATP, and of iodoacetate and fluoride, on the extra O_2 consumed after fructose addition. In each case the results were what would have been expected from the previous study of anaerobic metabolism: the addition of DPN stimulated the production of lactic and pyruvic acids in extracts incubated anaerobically, while it also stimulated the endogenous O_2 uptake of homogenates, the oxidation of fructose and of glucose-6-phosphate. On the other hand, inhibitors depressed both endogenous and fructose O_2 uptake, as well as the anaerobic accumulation of lactic and pyruvic acids. Lindberg & Ernster (1948) noted the stimulation of O_2 uptake after the addition of DPN to homogenates, but failed to observe any inhibition by iodoacetate. They interpreted these facts as showing that the glycolytic cycle was not functioning in their preparations and assumed that DPN was converted to TPN which then served as the coenzyme for the hexose monophosphate shunt.

The conversion of DPN to TPN occurs in certain yeast preparations (von Euler & Vestin, 1938; Kornberg, 1950). It seems unlikely that it takes place in egg homogenates of *Echinus esculentus*, since DPN addition has little or no effect on the oxidation of phosphogluconate, a TPN-catalysed reaction (Dickens, 1938*a*; Dickens & Glock, 1951).

The O_2 uptake of homogenates was stimulated by the phosphorylated intermediates of glycolysis. Apart from phosphogluconate, these intermediates have a comparable effect on aerobic and anaerobic metabolism. There was no disproportionate effect of glucose-6-phosphate as compared with hexose diphosphate, as described by Lindberg & Ernster (1948) in homogenates of the eggs of *Strongylocentrotus droebachiensis*. Indeed, the latter compound caused a greater stimulation than the former, something to be expected when the relative efficiency of these two substances is compared in glycolysis. A rigorous proportionality between the anaerobic and aerobic effects of these two substances is not to be expected, because the rate of oxidation of glycolytic pyruvate is a limiting factor which causes pyruvate accumulation.

In this work, the only observation which favours the existence of a functioning hexose monophosphate shunt is the oxidation of added phosphogluconate. This suggests that at least one enzyme of this system is present although it gives little quantitative indication about the relative 'strengths' of the two mechanisms. The immediate oxidation product of phosphogluconate—ribose-5-phosphate—might, by aldolase action, give rise to phosphoglyceraldehyde, so that the major part of the extra O_2 uptake found with phosphogluconate might be due to the glycolytic and tricarboxylic acid cycles. Experiments involving the addition of fluoride or iodoacetate could give more information on this point since the hexose monophosphate shunt itself is not affected by these substances. A more direct approach might be to determine the triose phosphate formed during the breakdown of glucose-6-phosphate, phosphogluconate and ribose-5-phosphate in iodoacetate-poisoned homogenates. This type of experiment and those in which DPN and TPN are added singly and together would probably enable one to make a more rigorous assessment of the relative importance of the glycolytic and oxidative mechanisms.

CONCLUSIONS

The experiments described in this paper have clearly established that the glycolytic cycle is functional during the aerobic metabolism of homogenates of the eggs of *Echinus esculentus*, and that the products of glycolysis can be completely oxidized. They also suggest that part at least of the hexose monophosphate oxidative pathway is present. Although the relative importance of the two mechanisms cannot as yet be quantitatively assessed, it is evident that the glycolytic mechanism is the more important. The main grounds on which these conclusions are based are recapitulated in Table 9.

Table 9

Evidence for glycolytic cycle in egg homogenates

1. Accumulation of pyruvate and lactate anaerobically and in presence of HCN.
2. Inhibition of O_2 uptake by fluoride and its reversal by pyruvate.
3. Accumulation of pyruvate after addition of glucose-6-phosphate.
4. No accumulation of pyruvate after addition of phosphogluconate.
5. Active oxidation of hexose diphosphate with pyruvate accumulation.
6. Stimulation of endogenous O_2 uptake by DPN.
7. Stimulation of fructose oxidation by DPN.
8. Inhibition of 6 and 7 by fluoride and iodoacetate.

Evidence for tricarboxylic acid cycle in egg homogenates

1. Active oxidation of cycle intermediates.
2. Complete oxidation of added pyruvate.
3. Inhibition of endogenous O_2 uptake by malonate and fluoracetate.

SUMMARY

1. The endogenous O_2 uptake of sea-urchin egg homogenates (*Echinus esculentus*) was inhibited by malonate, fluoride, iodoacetate, and fluoracetate.

2. The addition of the tricarboxylic acid cycle intermediates, citrate, α-ketoglutarate, succinate, fumarate, malate, and glutamate stimulated O_2 uptake.

3. The efficiency of the pyruvate-oxidizing mechanism varied in different preparations, but in some, added pyruvate was completely oxidized.

4. (1)–(3) strongly suggest the presence of a tricarboxylic acid cycle.

5. Glycogen, glucose and fructose stimulated O_2 uptake. The oxidation of fructose was stimulated by DPN and inhibited by fluoride and iodoacetate.

6. Glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and fructose-1:6 diphosphate stimulated O_2 uptake.

7. Phosphogluconate also stimulated O_2 uptake, which suggests that the enzymes of the hexose monophosphate shunt system may be present.

8. The experiments described in this paper show that the principal pathway by which carbohydrate is broken down is the normal glycolytic one.

One of us (R.) is indebted to the Medical Research Council for the provision of a laboratory assistant. Professor R. A. Peters, F.R.S., kindly gave us a sample of fluoracetate and Professor F. Dickens, F.R.S., gave us samples of phosphogluconic acid and also valuable advice.

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THE EFFECTS OF POSTERIOR PITUITARY EXTRACTS ON WATER BALANCE IN *BUFO CARENS* AND *XENOPUS LAEVIS*, TOGETHER WITH SOME GENERAL CONSIDERATIONS OF ANURAN WATER ECONOMY

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(*Received* 18 *December* 1951)

(With One Text-figure)

I. INTRODUCTION

In a previous communication (Ewer, 1951) it was noted that in both *Bufo regularis* and *B. bufo* pitressin has a greater water-balance effect than has pitocin, whereas in various species of *Rana* the reverse is the case. It was suggested that high pitressin sensitivity might be a characteristic of the genus *Bufo*, and that this might be correlated with the more terrestrial habit of toads, as compared with frogs. Before accepting this generalization as correct further data on other species of Anura are necessary; and a comparison of a terrestrial with an aquatic species also seemed desirable. *B. carens* occurs, although in small numbers only, in the neighbourhood, and is in general habit similar to *B. regularis*; the totally aquatic *Xenopus laevis* is also obtainable locally. The effects of pitressin and pitocin injection on water balance in these two species have therefore been investigated.

Other points in the physiology of the animals which were considered to be of interest in relation to their adaptations to terrestrial or aquatic life were also studied. These include the normal rate of water uptake, and the maximal rate of water uptake after desiccation of uninjected animals. It has further been found (Ewer, 1952) that in *Bufo regularis*, when the animals are kept in a damp atmosphere but without access to free water, that pituitrin injection causes resorbtion of fluid from the bladder and accumulation of fluid in the lymph sacs. The relative efficacy of pitressin and pitocin in causing these effects has now been investigated.

II. MATERIAL AND METHODS

The species used were *Bufo regularis* Reuss, *B. carens* Smith and *Xenopus laevis* (Daudin). The pituitary extracts used were Parke Davis pitressin, pitocin and pituitrin. All experiments were carried out at 26° C.

The method used for studying the effects of pitressin and pitocin injections on water uptake and urine output was the same as that described previously (Ewer, 1950, 1951). The rates of water uptake and urine production after injection of the pituitary extract are expressed as percentages of the normal rate, measured over a 3 hr. period immediately preceding injection. These values are referred to as the relative water uptake and relative urine production. The relative water uptake and urine production are calculated for the first hour after injection, and for the total period from the time of injection until the animal's weight has reached a maximum. In the case of *Xenopus* there is no weight gain after injection of the extracts, and the values have therefore been calculated for 1 hr. and for 2 hr. following injection.

The method of investigating the effects of pitressin and pitocin on lymph accumulation and fluid resorbtion from the bladder was as previously described (Ewer, 1952). The animal was first kept in water for 1 hr. to ensure a state of balance. The bladder was then emptied, the cloaca ligatured and the animal returned to water for a further 2 hr. The increase in weight at the end of this period has been found to be approximately equal to the weight of urine in the bladder. The animal was now injected with the appropriate extract, and was placed in a moist chamber for 2 hr. At the end of this period it was killed by decapitation, and the fluid in the femoral lymph sacs and in the body cavity and also the urine in the bladder were collected and weighed. The weight of lymph is expressed as a percentage of the animal's original weight, while the quantity of urine resorbed from the bladder is expressed as a percentage of the bladder content at the time of injection, and also as weight resorbed per hour per 100 g. body weight.

III. RESULTS

(1) *The effects of pitressin and pitocin on water uptake and urine output in* Bufo carens *and* Xenopus laevis

With *Bufo carens* experiments have been carried out at two dosages, 1 and 0·1 i.u./100 g. body weight. Only a small number of specimens could be obtained, and therefore only three sets of experiments at each dosage were carried out. The results are given in Tables 1 and 2. Since there are only three readings the standard errors of the means cannot be used in assessing the significance of the differences found between the effects of pitressin and pitocin. The values for the two doses may, however, be considered together, and the variance analysed. When this is done pitressin is found to have a significantly greater effect than pitocin upon relative water uptake (F 1/8 = 5·99), relative urine production (F 1/8 = 6·43) and maximum weight increase (F 1/8 = 15·72). These results closely resemble those previously obtained with *B. regularis* (Ewer, 1950).

In the case of *Xenopus laevis* experiments were carried out at dosages of 2·5 and 0·25 i.u./100 g. body weight. The results are given in Tables 3 and 4. The injections were not followed by any consistent anti-diuretic effect, and indeed the urine flow usually increased after injection, keeping pace with the increased rate of water uptake, so that there was no increase in weight. Pitressin and pitocin both cause an increase in rate of water uptake, although the responses are considerably smaller than those shown by the two species of *Bufo*. The difference found between the effects of pitressin and pitocin are not significant at either dosage. In responding approximately equally to the two extracts *Xenopus* differs from any of the species of either *Bufo* or *Rana* which have been investigated to date.

Table 1. *The effects of pitressin injection on* Bufo carens. *Each row of figures gives the results for a single animal*

Dosage (i.u./100 g.)	Relative water uptake		Relative urine production		Duration of anuresis (hr.)	Max. wt. gain (%)
	1st hr.	Total period	1st hr.	Total period		
1·0	376	248	5·4	23·6	2	30·4
	206	149	36·7	34·4	2	13·8
	376	189	16·4	32·4	1	22·2
Mean	319	195	19·5	30·1	1·7	22·1
0·1	198	142	17·1	29·0	1	9·0
	150	119	22·3	38·9	1	9·0
	277	—	16·8	—	1	13·9
Mean	208	131	18·7	34·0	1	10·3

Table 2. *The effects of pitocin injection on* Bufo carens. *Each row of figures gives the results for a single animal*

Dosage (i.u./100 g.)	Relative water uptake 1st hr.	Relative urine production 1st hr.	Duration of anuresis (hr.)	Max. wt. gain (%)
1·0	184	54·7	0	6·2
	184	21·1	1	9·3
	287	87·0	0	9·6
Mean	218	54·3	0·3	8·3
0·1	106	45·4	0	3·6
	131	49·8	0	3·2
	107	26·7	1	4·6
Mean	115	40·6	0·3	3·8

Table 3. *The effects of pitressin injection on* Xenopus laevis. *Each figure represents the mean of seven experiments*

Dosage (i.u./100 g.)	Relative water uptake		Relative urine production		Duration of anuresis
	1st hr.	1st 2 hr.	1st hr.	1st 2 hr.	
2·5	129·0 ± 7·54	124·3 ± 6·85	103·6 ± 24·70	116·1 ± 21·20	0
0·25	112·7 ± 3·35	105·9 ± 3·54	104·4 ± 23·56	109·0 ± 22·47	0

Table 4. *The effects of pitocin injection on* Xenopus laevis. *Each figure represents the mean of seven experiments*

Dosage (i.u./100 g.)	Relative water uptake		Relative urine production		Duration of anuresis
	1st hr.	1st 2 hr.	1st hr.	1st 2 hr.	
2·5	124·8 ± 8·82	120·9 ± 6·31	134·9 ± 16·31	116·9 ± 11·30	0
0·25	117·7 ± 8·18	118·4 ± 6·22	162·5 ± 15·94	161·8 ± 11·58	0

(2) *The effects of pitressin and pitocin on fluid distribution in* Bufo regularis

Some preliminary experiments suggested that the lymph accumulation response might be seasonably variable. Experiments were therefore carried out on three pairs of animals at the end of each month from April to October. The results are given in Table 5. The individual variation is very great, but pitressin gives consistently higher values, both for the weight of lymph accumulated and the resorbtion of urine. There are indications that the responses are least marked in winter (June–August) and increase once spring has begun, and the animals emerge from their winter hiding and are active again. The maximum is reached at the end of May. There are, however, not sufficient data to lay any great stress on the seasonal changes. The values for all months may be summed, the means calculated and the significance of the differences between the effects of pitressin and pitocin assessed by the t test. When this is done the difference in total fluid accumulated is found to be just significant ($P \bumpeq 0{\cdot}2$), while the difference in urine resorbtion is highly significant ($P < 0{\cdot}001$). A better comparison may be made by making an analysis taking into account the variation from month to month. When this is done a value of $F\ 1/20 = 8{\cdot}16$* is found for total lymph accumulation. This is significant at the 1 % level. The significance of the variation between months may also be tested, and gives a value of $F\ 4/20 = 7{\cdot}50$, which is also significant at the 1 % level. Thus in *Bufo regularis* pitressin is more effective than pitocin in causing lymph accumulation and urine resorbtion. This is in harmony with its greater water-balance effect in this species.

Table 5. *The effects of pitressin and pitocin on lymph accumulation and urine resorbtion in* Bufo regularis. *Each figure is the mean of three determinations made at the end of the month indicated. The columns headed 'Press.' and 'Oxy.' give the values found after injection of pitressin and pitocin respectively*

Date	Fluid in lymph sacs and body cavity (% body wt.)		Resorbtion of urine (% original bladder content)		Resorbtion of urine (% body wt./hr.)	
	Press.	Oxy.	Press.	Oxy.	Press.	Oxy.
Apr.	2·42	2·10	100	83·9	3·26	2·23
May	5·93	3·40	99·0	65·1	4·45	2·35
June	2·76	2·52	97·2	93·5	3·11	3·28
July	1·74	1·40	89·0	72·6	2·22	2·03
Aug.	3·23	2·41	100	63·5	3·64	2·44
Sept.	4·53	2·36	100	92·5	3·51	3·50

A few qualitative experiments were performed on *B. carens*. This species also shows the lymph accumulation response after pituitrin injection. Out of seven animals injected with pituitrin at a dosage of 3 i.u./100 g. body weight four showed large distension of the lymph sacs, two gave a definite but smaller response, and in one the response was doubtful.

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* The values for the end of August have had to be omitted in this calculation, since in this series the weight of the lymph in the body cavity of one animal could not be measured owing to contamination with blood.

(3) *The normal rates of water uptake*

The rate of water uptake, expressed as percentage body weight per hour, varies with the size of the animal. Small specimens, with a relatively greater surface area, have naturally the highest rates of water uptake. The rates of uptake per unit surface area would be expected to be more constant, but since conversion factors have not been worked out for the species under consideration the values are given as per 100 g. body weight per hour, and are grouped into various weight classes. The results are given in Table 6 and Fig. 1. The rate of water uptake of *B. regularis* is significantly greater than that of *Xenopus laevis*, while the single value for *Bufo carens* does not differ significantly from the value for *B. regularis* of the same weight.

Table 6. *The normal rates of water uptake of the three species of Anura indicated. Each figure is a mean value for the number of animals given in the third column*

Bufo regularis			*Bufo carens*			*Xenopus laevis*		
Wt. class (g.)	Uptake (%/hr.)	Number	Wt. class (g.)	Uptake (%/hr.)	Number	Wt. class (g.)	Uptake (%/hr.)	Number
20–30	7·8 ± 0·66	9	30–50	5·65 ± 0·18	12	Up to 25	2·4 ± 0·12	17
31–35	6·9 ± 0·49	8				26–50	1·9 ± 0·09	12
36–40	6·5 ± 0·36	15				70 and over	1·6 ± 0·08	9
41–45	5·7 ± 0·38	11						
46–50	4·9 ± 0·26	12						
51–60	5·0 ± 0·26	12						
61–80	5·0 ± 0·41	11						
81–100	4·1 ± 0·32	7						
Over 100	3·7 ± 0·38	9						

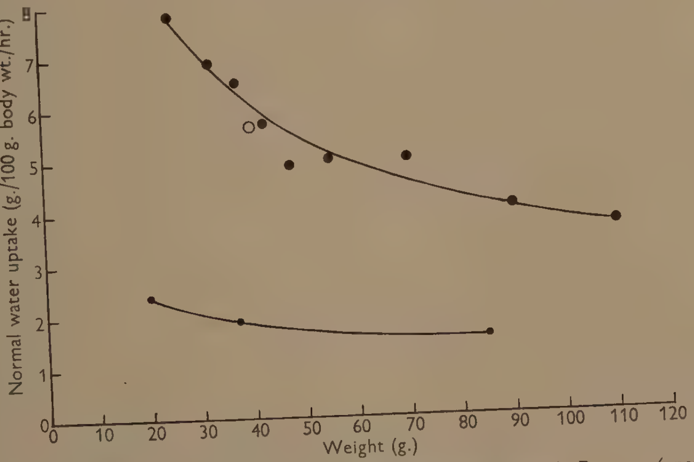

Fig. 1. Normal rates of water uptake of *Bufo regularis* (large filled circles), *B. carens* (open circle) and *Xenopus laevis* (small filled circles).

It is difficult to compare these values with those of other workers, since the experimental temperatures have not been the same. This difference between more terrestrial and more aquatic species has, however, been noted previously. Rey (1937) gives values of 22, 9 and 5 mg./cm.^{2}/hr. for *B. vulgaris* Laur. = *B. bufo* (L.), *Rana temporaria* L. and *R. esculenta* L. respectively, while Jørgensen (1950) finds the rate of *Bufo bufo* to be about twice that of *Rana temporaria*.

(4) *The rate of water uptake after desiccation*

In § (2) it was noted that the response of *Xenopus* to posterior pituitary extracts was slight compared with those of the two species of *Bufo*. It was thought that the two genera might also show a difference in their ability to take up water after they had been subjected to desiccation. Experiments to test this point were therefore carried out on *B. regularis* and *Xenopus*. To ensure full hydration the *Bufo* were kept in water for an hour before an experiment was started; *Xenopus* is, of course, normally kept in water. The animal was weighed, its bladder having first been emptied by means of a cannula. It was then left in a wire-covered dish exposed to the draught of an electric fan until it had lost between 10 and 20 % of its original weight. It was then replaced in water and weighed again after half an hour, after 1 hr. and after 2 hr. or until it had regained its original weight. The rate of water uptake is expressed as the weight increase per 100 g. body weight per hour and can be compared with the normal rate of uptake of an animal of the same weight found from Fig. 1. The results are given in Table 7. The two genera behave very differently. *Bufo regularis* after desiccation is capable of taking up water at a remarkable rate, the highest value found being 36 % per hour, representing more than seven times the normal rate. *Xenopus*, on the other hand, shows very little ability to increase its rate of water uptake; in only three out of the seven experi-

Table 7. *The rates of water uptake after desiccation of* Bufo regularis *and* Xenopus laevis

Species	Original wt. (g.)	Desiccation (% wt. lost)	Uptake in 1st ½ hr.		Uptake in 2nd ½ hr.		Uptake in 2nd hr.	
			%/hr.	% normal	%/hr.	% normal	%/hr.	% normal
Bufo regularis	62·9	18·9	29·5	604	15·2	242		
	61·7	16·0	36·0	734	—	—		
	119·4	16·0	23·3	647	7·3	203		
	48·5	14·8	16·8	305	15·4	281		
	81·4	14·4	24·2	570	17·7	418		
	113·4	11·5	20·9	550	—	—		
	94·2	9·8	13·2	348	6·2	158		
	Means	14·5		534		260		
Xenopus laevis	26·8	15·4	2·77	123	2·62	111	2·36	105
	19·7	14·5	3·05	127	2·95	123	2·95	123
	128·5	12·8	1·34	89	1·11	74	1·21	81
	42·9	12·4	1·87	104	2·19	122	—	—
	131·7	11·7	1·26	84	0·90	60	1·00	67
	26·1	11·6	1·30	59	1·68	76	1·65	75
	148·2	10·4	1·41	94	—	—	1·34	89
	Means	12·7		97		95		90

ments was there any increase, the maximum being 127 % of the normal rate. The decrease shown in the other experiments is probably due to the drying of the sticky mucous secretion on the surface of the animal, which may hinder water uptake. These data strongly suggest that in *Bufo regularis* the high rate of water uptake after desiccation is not a passive phenomenon, brought about solely by the increase in the osmotic pressure of the body fluids, but very probably involves the secretion of water-balance principle from the posterior lobe of the pituitary. It would be of considerable interest to repeat these experiments on hypophysectomized animals.

(5) *The maximal weight increase*

In most of the early studies of the effects of posterior pituitary extracts on anuran water balance no distinction was made between renal and dermal effects, and the net weight increase alone was measured. This weight increase after injection of posterior pituitary extracts is known as the Brunn reaction. The magnitude of the Brunn reaction is obviously a function of both the renal and dermal responses, since unless there is an antidiuretic response an increase in water uptake would not result in a weight increase, as the excess water would merely be excreted by the kidney. Steggerda (1937) has pointed out that there appears to be a correlation between the magnitude of the Brunn effect and the habits of the animal, the more terrestrial species showing the larger responses. With this view the present findings are in full accord; the totally aquatic *Xenopus* shows no weight increase, while the two species of *Bufo* have large Brunn reactions. Table 8 summarizes the available data on the Brunn reaction, and these in general support Steggerda's conclusion. Jørgensen (1950), in discussing this question, is doubtful whether the correlation between Brunn reaction and degree of terrestrial adaptation is valid, since the findings of certain workers seem to contradict it. These cases, however, require further consideration. Novelli (1933) finds a weight increase of 18 % in *B. arenarum* Hensel, and 24–28 % in *Leptodactylus occelatus* (L.). These values cannot be accepted, since Novelli made his first weighings 10 hr. after injection, and there is no indication in his work of his having previously determined that the weight is at a maximum at this period. Other workers have generally found that species of *Bufo* show their maximal weight gain 4 or 5 hr. after injection, and Novelli's value of 18 % is therefore probably not the maximum weight attained. Bělehrádek & Huxley's (1927) experiments with *Amblystoma tigrinum* are too scanty to be of any value. Jørgensen further quotes Howes's (1940) value of 14 % weight increase in newly metamorphosed *Bufo bufo*, but does not mention that in fully adult specimens Howes found a value of 40 %.

The values of 22 % for the two species of *Bufo* found in the present work are low for this genus. This is probably due to the fact that the experimental temperature was high, namely 26° C. Boyd & Brown (1938) have shown that the magnitude of the Brunn reaction varies with temperature, being less at higher temperatures. Unfortunately, few workers have stated the temperatures at which they worked; and the very different values found for *Rana temporaria* by Brunn (1921) and Rey (1935) may possibly be due to their having worked at different temperatures.

IV. DISCUSSION

The effects of posterior pituitary extracts on anuran water balance may be considered from two aspects: their bearing on the identity of the active principles of the anuran posterior pituitary, and the biological significance of the responses.

The oxytocic and pressor fractions of mammalian posterior pituitary extract are chemically similar; Waring & Landgrebe (1950) state that 'it seems likely that the specific activities of the posterior lobe are due to closely related polypeptides of molecular weight about 2000'. Since most previous work has been performed on species of *Rana* there has developed a tendency to assume that the amphibian water-balance principle is a single entity, and that it is most closely related to the oxytocic fraction of mammalian posterior pituitary extract. Heller (1950) defines amphibian water-balance activity in terms of the effects on *Rana*, but points out the desirability of investigations on more species of Anura. The results of the present investigation show that the Anura vary in their sensitivities to the oxytocic and pressor fractions of mammalian extracts; while *Rana* is most sensitive to the oxytocic fraction, it has now been shown that the genus *Bufo* is most sensitive to the pressor fraction, while *Xenopus* responds equally to both. In addition Jørgensen (1950) has shown that extracts of *Rana temporaria* and *Bufo bufo* pituitaries differ in their anti-diuretic potencies whichever species is used as the test animal. These data suggest that

Table 8. *The magnitude of the Brunn reaction in various species of Amphibia*

Species	Maximum wt. gain (%)	Temp. (° C.)	Author
Necturus maculatus Baird	*c.* 4	Not stated	Steggerda (1937)
Amblystoma mexicanus (Shaw) (larva)	3·3	Not stated	Schreiber & Schreiber (1951)
Amblystoma mexicanus (metamorphosed)	7	Not stated	Schreiber & Schreiber (1951)
Bufo bufo (L.) (young tadpoles)	0	Not stated	Howes (1940)
Bufo bufo (L.) (older tadpoles)	5–10	Not stated	Howes (1940)
Xenopus laevis (Daudin)	0	26	Ewer (present paper)
Bufo bufo (L.) young toads	10	Not stated	Howes (1940)
Rana temporaria L.	28	Not stated	Brunn (1921)
Rana temporaria L.	5–10	Not stated	Rey (1935)
Rana pipiens Schreiber	*c.* 20	15	Boyd & Brown (1938)
Rana pipiens Schreiber	*c.* 14	25	Boyd & Brown (1938)
Rana pipiens Schreiber	*c.* 8	35	Boyd & Brown (1938)
Rana pipiens Schreiber	18	Not stated	Steggerda (1937)
Rana pipiens Schreiber	19	Not stated	Sawyer, Travis & Levinsky (1950)
Rana catesbiana Shaw *Rana septentrionalis* Baird	Said not to differ significantly from *Rana pipiens*		Boyd & Brown (1938)
Rana clamitans = *Rana clamata* Daudin	14	Not stated	Steggerda (1937)
Rana esculenta L. (winter frogs)	12	Not stated	Heller (1930)
Rana esculenta L. (summer frogs)	24	Not stated	Heller (1930)
Leptodactylus ocellatus (L.)	16	Not stated	Biasotti (1923)
Bufo americanus Harlan	45	Not stated	Steggerda (1937)
Bufo bufo (L.)	*c.* 40	Not stated	Howes (1940)
Bufo regularis Reuss	22	26	Ewer (1951)
Bufo carens Smith	22	26	Ewer (present paper)

within the Anura the water-balance principle is not identical in different genera but that in all cases the chemical resemblance to pitocin and pitressin is close. In some genera the animal's own water-balance principle may be assumed most closely to resemble the pressor fraction, while in others the resemblance is closer to the oxytocic fraction of mammalian extract. The greater response will be expected to be shown to the mammalian extract which most closely resembles the animal's own water-balance principle. Which fraction evokes the greater response in any particular genus cannot therefore be regarded as having any significance other than indicating its relationship to the secretions of the posterior pituitary in the genus concerned. Heller & Smith (1948) obtained an extract from crabs' eye stalks which caused an increase in weight in *Rana temporaria*, but had neither oxytocic nor anti-diuretic effects in mammals. In this case we must assume that the eye-stalk extract sufficiently resembled the natural water-balance principle of *Rana* to have an effect, but was not sufficiently similar to the mammalian hormones to be effective in this class.

The two species of *Bufo* which have been investigated are highly adapted to terrestrial life, and can exist for long periods without access to ponds or streams. A comparison of the water economy of these species with the less terrestrial *Rana* and the aquatic *Xenopus* may therefore be expected to throw some light on the physiological adaptations of Anura to life on land. The normal rate of water uptake in *Bufo* is extremely high, and becomes even higher after desiccation, whereas that of *Xenopus* is low and shows little or no increase after desiccation. The terrestrial toad can therefore readily absorb free water when this becomes available and can take advantage of temporary supplies provided by rain and dew. Such a high normal rate of uptake would, however, be unsuited to an aquatic form; it would merely throw an increased amount of work on the kidneys, since the water would have to be pumped out as fast as it entered.

In its responses to pituitary extracts *Bufo* shows a number of characteristics which appear to be correlated with its terrestrial habit. The increase in water uptake following injection is very considerable, and there is a well-marked anti-diuretic effect. As a result there is a large weight increase, the animal being able not only to take up water rapidly, but to hold an increased amount of fluid for some time. This extra fluid, as has previously been shown, is held mainly in the lymph sacs (Ewer, 1950). This accumulation of fluid in the lymph sacs will take place after injection of posterior pituitary extracts even if the animal has no access to external supplies of water. In these circumstances if the bladder be full, the fluid in it can be resorbed, thus providing a further method of conserving water.

Heller (1950) points out that the water-conserving role of the secretions of the posterior pituitary constitutes an example of the way in which, within the different classes of the vertebrates, a hormone may perform a similar function by acting on morphologically non-homologous effectors. In mammals water conservation is achieved by increased resorbtion in the kidney tubule. In the Amphibia the anti-diuretic effect appears to be brought about by constriction of the afferent glomerular arterioles (Heller, 1950), while increase in water absorbtion occurs not in the kidney, but in the skin. In addition, it has now been shown that in *Bufo* the water-conserving mechanisms include the resorbtion of fluid from the bladder and the regulation of the volume of the lymph. Whether this latter is achieved by effects on the smooth muscle of the lymph hearts, or by changes in capillary permeability or blood pressure, is not yet clear. It may, however, be pointed out that the converse of Heller's statement also holds: to wit, that the same secretions in different classes of vertebrates may, by acting on morphologically similar structures, fulfil diverse functional roles. In the Anura the effects of posterior pituitary secretions on smooth muscle (of renal blood vessels and possibly lymph hearts) assist in water conservation, while in mammals the smooth muscle responses are concerned with the regulation of blood pressure and the physiology of the pregnant uterus.

V. SUMMARY

1. The effects of pitressin and pitocin on water balance in *Bufo carens* and *Xenopus laevis* have been investigated. *Bufo carens* is most sensitive to pitressin, and shows an increased water uptake together with a well-marked anti-diuretic response. *Xenopus* reacts equally to the two extracts by an increase in water uptake, but there is no anti-diuresis.

2. The effects of pitressin and pitocin in causing lymph accumulation and resorbtion of fluid from the bladder have been studied in *Bufo regularis*. Pitressin has the greater effect, and there are indications of seasonal variations in the magnitude of the response.

3. The normal rates of water uptake of *B. regularis*, *B. carens* and *Xenopus laevis* have been measured. The two species of *Bufo* take up water rapidly, but *Xenopus* does so much more slowly. After desiccation the water uptake of *Bufo regularis* increases very considerably. This does not occur in *Xenopus*.

4. There is a large weight increase after injection of posterior pituitary extracts in *Bufo regularis* and *B. carens*, but not in *Xenopus laevis*. The findings of other workers on the magnitude of the weight increase after posterior pituitary injections are summarized.

5. The results are discussed in relation to the identity of the active principles of the anuran posterior pituitary and to the biological significance of the responses.

This work was carried out during the tenure of a Research Grant from the South African Council for Scientific and Industrial Research.

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THE COMPOSITION OF THE BODY FAT OF SMALL GREEN CHIRONOMIDS

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(*Received* 13 *October* 1951)

During the winter months, the banks of the Nile near Khartoum abound with small non-biting midges, *Tanytarsus lewisi* Freeman (Diptera, Chironomidae), which emerge from the surface of the river shortly after sunset and fly around in the immediate neighbourhood for about 1½ hr. In an endeavour to relate variations in the incidence of these insects ('nimitti') to seasonal and climatic factors, collections were made every evening for some considerable time; the insects trapped for this purpose provided the source of material used for the experimental work described below.

The trap used, which was designed by Dr A. J. Henry, Government Analyst, consisted of a wooden box, 56 × 56 × 45 cm., which was placed on a table about 4 ft. high, situated on a first-floor verandah of a building on the south bank of the Blue Nile at Khartoum. One side panel (56 × 56 cm.) was removed, and the whole box was painted dull black, inside and out. A metal can, 23 cm. deep and 18 cm. in diameter, lined with black paper inside, was placed centrally on the bottom of the box, the open end uppermost, and a high-powered electric light bulb was suspended just above the top of the can. This was illuminated each night from 6.30 to 8 p.m. when the insects would swarm around the lamp and fall down into the can. Once inside the can it seems impossible for them to escape, and it was furthermore noticed that practically no insects fell outside it. The next morning the dead insects were dried for 5 hr. in a hot-air oven at 50° C. and weighed. Approximately 18,000 insects weighed 1 g. and as many as a million have been caught by this procedure in one evening.

The air-dried insects were analysed with the following results:

	%
Loss at 110° C.	4·39
Ash	5·64
Protein	60·68
Chloroform-soluble fraction	11·71
Alcohol-after-chloroform extract	17·78
	100·20

EXAMINATION OF THE CHLOROFORM-SOLUBLE FRACTION

This was a very dark brown evil-smelling oil, having the following characteristics:

Free fatty acids (as oleic acid)	22·28 %
Saponification value	205·5 mg. KOH/g.
Unsaponifiable matter	7·45 %

The figure for the saponification value must be regarded as approximate only, as the dark colour of the mixture obscured the end-point very considerably. The unsaponifiable matter, removed in the usual way by extracting the saponified oil with ether, was golden yellow in colour, had a pleasant fragrant smell and tended to crystallize radially on standing. The alkaline solution after extraction was acidified and the fatty acids were recovered by re-extraction with ether. This fraction represented 88·38 % of the whole and had the following characteristics:

Iodine value (Wijs) (1½ hr.)	137·2
Thiocyanogen value (20 hr.)	93·02
Mean molecular weight	280

BROMINATION OF TOTAL NON-VOLATILE FATTY ACIDS

0·90 g. of this fraction was dissolved in 20 c.c. of ether and cooled to 0° C. Bromine was added drop by drop, and a white precipitate appeared. This was allowed to stand in a refrigerator for 24 hr., when it was centrifuged and the mother-liquors carefully decanted. The deposit was stirred up with a further 20 c.c. of ice-cold ether, and again centrifuged and decanted. The residual crystals weighed 0·1594 g. (17·7%). On attempting a melting-point determination, no sharp melting-point was obtained below 210° C., when decomposition with blackening occurred. This compound is not, therefore, the bromination derivative of linoleic acid, but is derived from a tetra-ene or penta-ene acid similar to those present in fish oils.

The mother liquors which had been decanted from these crystals were evaporated to dryness on a water-bath, and treated with hot petroleum ether. An insoluble heavy oily fraction was obtained, and on cooling the petroleum ether, a crop of colourless crystals was also obtained. After twice recrystallizing from petrol ether, this compound had m.p. 114° C., and was evidently the bromination product of linoleic acid present in the original oil.

IDENTIFICATION OF THE LESS UNSATURATED FATTY ACIDS

5 g. of the original oil were subjected to the usual Reichert-Meissl-Polenske process, after saponification with glycerolic sodium hydroxide in the usual way. The Reichert-Meissl value was 1·91 and the Polenske value 0·52.

The distillates from the Reichert-Meissl and Polenske determinations were evaporated to dryness, and the following residues were obtained:

Reichert-Meissl:

Total residue	0·0256 g.
Phenolphthalein added	0·0050 g.
Weight of sodium salt of water-soluble volatile acids	0·0206 g.

This was equivalent to 1·74 c.c. N/10-NaOH (titration figure for Reichert Meissl value).

The molecular weight of the water-soluble steam-volatile acids is therefore 96 (butyric acid 88, valeric acid 102).

Percentage water-soluble volatile acids present: 0·37 %.

Polenske:

Total residue	0·0168 g.
Blank on indicator and alcohol	0·0028 g.
Weight of sodium salts of Polenske acids	0·0140 g.

This was equivalent to 0·52 c.c. N/10-NaOH, corresponding to a molecular weight of 247 for the Polenske acids, which was present to the extent of 0·26 %. It is considered that this finding has no real significance, being due in all probability to a small portion of the non-volatile acids having been carried over by mechanical splashing.

The residue from the Reichert-Meissl-Polenske distillation flask was recovered by extraction with ether, and 4·7802 g. were obtained (95·60 %). This material was saponified by refluxing with excess N/2 alcoholic caustic potash for 1 hr., and the unsaponifiable matter extracted; 0·3748 g. (7·496 %) was obtained. The extracted soap solution was acidified and the liberated fatty acids were recovered by ether extraction (88·10 %). This had a mean molecular weight of 280·0.

MILD OXIDATION OF TOTAL NON-VOLATILE FATTY ACIDS

These non-volatile fatty acids were oxidized under the conditions described by Lapworth & Mottram (1925). 3·50 g. fatty acids were taken and dissolved in 1400 c.c. water containing 3·5 g. of caustic soda. 490 g. of ice were added to the mixture and then a solution of 5·32 g. potassium permanganate dissolved in 250 c.c. of water, cooled in ice to 0° C. The addition of the solution took 3 min., and the reaction mixture allowed to stand for a further 12 min., the whole being kept thoroughly cooled with ice. In this way it was hoped to produce only hydroxylation of the unsaturated fatty acids and avoid any oxidative split to dibasic acids and short-chain monobasic acids. The mixture assumed the brilliant green colour of manganate, which was finally decolorized by the passage of sulphur dioxide. The liberated acids were allowed to stand overnight in the refrigerator to coagulate. The mixture was then filtered at the pump, and the filtrate extracted with petrol ether. The residue was transferred to a beaker, and boiled up thoroughly with the same petrol ether, the solution being filtered into a separating funnel, washed with water until neutral, and evaporated. The residue was a hard brittle fat (1·1904 g.) equivalent to 34·0 % of the total non-volatile fatty acids. This had a molecular weight of 271·9, and m.p. 54° C., and was evidently mainly stearic acid in admixture with small amounts of palmitic acid.

The residue insoluble in petrol ether was boiled up with about 1200 c.c. of water and filtered hot at the pump, a further 800 c.c. boiling water being used for washing. The filtrate and washings were united and evaporated to about 1 l. and then cooled in the refrigerator overnight. The deposited crystals were filtered off at the pump, and washed with ice-cold water. 0·6631 g. was obtained, and a further 0·0998 g. was recovered by evaporating the filtrate to dryness. By repeated recrystallization from ethyl acetate, two compounds were isolated, melting at 148° C. (more-soluble fraction) and 172° C. (less-soluble fraction), and had molecular weights 345 and 351 respectively. These compounds are therefore the well-known pair of isomeric tetrahydroxystearic acids corresponding to *cic-cis*-9, 12, octadecadienoic acid—the linoleic acid commonly found in seed fats, and whose presence in this insect fat is thus established.

The water-insoluble residue was digested with boiling ethyl acetate, filtered, concentrated and cooled. A white crystalline deposit was obtained which was further recrystallized twice from ethyl acetate, in which it was readily soluble at the boil, but had a low solubility at 0° C. This compound had m.p. 116° C. and molecular weight 313, and was evidently dihydroxystearic acid probably contaminated with a small amount of dihydroxypalmitic acid, arising from the mild oxidation of oleic acid (and minor amounts of palmitoleic acid) in the original mixed fatty acids.

The fatty material contained in these insects is therefore probably a mixture of glycerides of palmitic, stearic, oleic, palmitoleic, linoleic acids and a polyethenoid acid containing four or five double bonds, and although no quantitative estimate of the composition is given here, the fat is clearly closely similar to those usually occurring in fresh-water fish (Lovern, 1932 et seq.).

It is interesting to note that in all probability the eggs of these insects are laid on the surface of the water, and sink to the bottom where they hatch out in the mud. The larvae spend most of their time in the mud, in which they feed on algae and other similar materials. The pupae, which do not feed, remain on the bottom until they rise to the surface preparatory to emerging as adult insects, which also do not feed, and have only a very short life, probably only a few hours. Thus virtually all the feeding takes place in the aquatic phase. As the insects of course do not return to the water, their emergence represents a considerable loss of protein and fat from river circulation every year. The insects form an important source of food material for the fish, the composition of the fat of which is so closely similar to that of the insects, which in turn resembles that present in algae (Lovern, 1936), all three types containing notable proportions of polyethenoid acids.

SUMMARY

The body fat of the midge, *Tanytarsus lewisi* (Chironomidae), has been analysed, and has been found to contain major amounts of polyethenoid fatty acids normally associated with the fat of fish. It is interesting to note that this insect does all its feeding while in the larval stage on the bed of the river, under water, and that the body fat resembles that of algae, and plankton, which probably are the main sources of food, and differs considerably from the composition of most terrestrial insects hitherto recorded.

The author wishes to thank Dr A. J. Henry, Government Analyst, for collecting the insects and suggesting this investigation; Mr D. J. Lewis and Dr J. Rzoska for helpful criticism; and the Director, Medical Services, for permission to publish this paper.

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THE MORPHOLOGY OF BULL SPERMATOZOA

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(*Received* 9 *January* 1952)

(With Plates 17–19 and One Text-figure)

INTRODUCTION

It has been known since the nineteenth century that the microscopical structure of living cells may be modified by fixation and by post-mortem or sub-mortem changes (Hardy, 1899), but inadequate attention seems to have been paid to factors which may injure spermatozoa before fixation and microscopical examination. One such factor is the effect of sudden cooling.

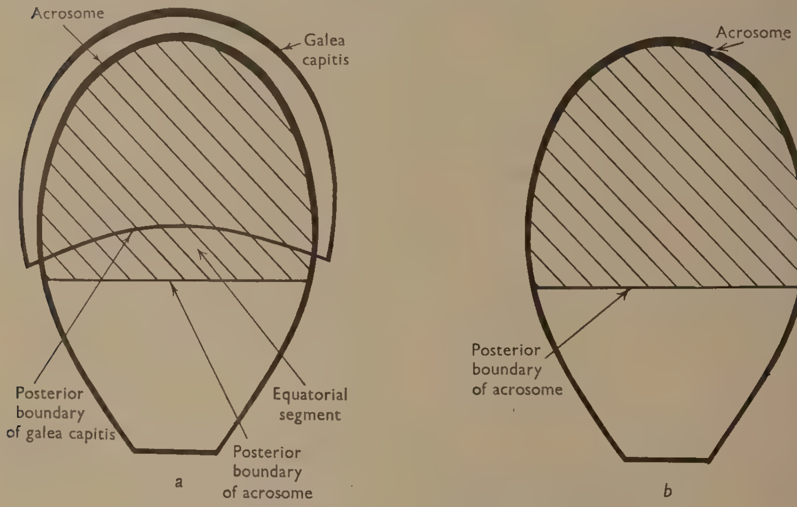
If semen is exposed to a sudden fall in temperature there is an irreversible decline in the activity of the spermatozoa (Chang & Walton, 1940), and this reduction in activity is associated with an increase in the number of dead, i.e. eosinophil cells (Lasley, Easley & McKenzie, 1942). This phenomenon of 'temperature shock' is encountered in bull semen cooled abruptly by pipetting semen at 30° C. into an aqueous solution of nigrosin and eosin at 20° C. (Hancock, 1951). The depressed rate of respiration and fructolysis of temperature-shocked samples can be correlated with the increase in the proportion of eosinophil cells (Bishop & Hancock, 1951).

When bull spermatozoa are stained to differentiate living from dead spermatozoa, the acquisition of staining affinity by the dead spermatozoa is associated with obvious structural changes. These changes are likely to be encountered in fixed specimens unless special precautions are taken to avoid lethal damage from 'temperature shock' before fixation.

The introduction of the electron microscope has given a new impetus to the study of the morphology of spermatozoa. The spermatozoa of the bull have been studied and described in great detail (Bretschneider & van Iterson, 1947; Bretschneider, 1949, 1950) and Randall & Friedlaender (1950) have used the method to investigate the structure of ram spermatozoa. The published results of these studies contain, however, no observations on the need for special precautions in the treatment of spermatozoa before fixation, and it is possible that some of the structural features which have been described may be absent from the intact living cell and appear only as artifacts after its death.

In this paper the morphological features of living and dead bull spermatozoa are described. The differences which have been observed relate to the structure of the head. It is therefore convenient at this stage to record the principal structural features which have been described in the head of the bull spermatozoon. These are illustrated diagrammatically in Text-fig. 1*a*.

The galea capitis is shown as a loose cap investing the anterior half of the head and overlying a second more compact cap, the acrosome, which is closely applied to the head. The posterior boundary of the galea capitis marks the anterior border of the equatorial segment which is bounded posteriorly by the posterior margin of the acrosome. This reconstruction of the head of a bull spermatozoon is based on a number of sources discussed throughout this paper. It should be compared with the head shown in Text-fig. 1*b*. Here, the head is covered by a single structure called the acrosome. This is the 'cap' referred to throughout this paper. Text-fig. 1*b* is based on the experiments described below, which deal with the variations in the morphological appearance of the acrosome; the appearance of the acrosome in Text-fig. 1*b* is characteristic of the living spermatozoon.


Acrosome
Galea capitis
Posterior boundary of galea capitis
Equatorial segment
Posterior boundary of acrosome
a
Acrosome
Posterior boundary of acrosome
b

Text-fig. 1. *a*. Conventional diagram of head of bull spermatozoon. *b*. Head of living spermatozoon (see text).

MATERIALS AND METHODS

Semen was obtained from four bulls at the Animal Research Station, Cambridge, and twelve animals at the Cambridge Cattle Breeding Centre. It was collected in an artificial vagina and held in a water-bath at 30° C. before any subsequent treatment.

To preserve the vitality of samples selected for study of the characteristics of the living cell, ejaculates were maintained at 30° C. during examination and fixation. These operations were usually completed within 30 min. of collection. When examination was delayed the samples were placed in a 100 ml. beaker containing water at 30° C. and transferred to a refrigerator at 4° C. so that cooling to this temperature occupied not less than 1 hr., and temperature shock was avoided.

The characteristics of the dead cell were studied in spermatozoa killed by cooling rapidly to 0 or −10° C., and in samples of semen cooled, with precautions against temperature shock, to 4° C. and stored at this temperature for 2–3 weeks. Additional observations were made on the dead spermatozoa encountered in varying proportions in freshly ejaculated semen.

The proportions of live and dead cells were determined, for all samples studied, at the time of collection and at the time of examination or fixation. It was thus possible to relate the cytological characteristics of test populations to their viability as determined by the differential staining technique. The following techniques were used:

Nigrosin-eosin stain. This was prepared by dissolving 5 g. water-soluble eosin Y (Gurr) in 300 ml. of a 10 % solution of nigrosin (Gurr) in distilled water.

The stain was used to differentiate living and dead spermatozoa and for the preliminary study of their cytological differences (Hancock, 1951). The number of living cells was determined in suspensions prepared by diluting one drop of fresh semen at 30° C. in eight drops of the stain at this temperature. After 5 min. one drop of the suspension was smeared on to each of two slides on a warm stage, allowed to dry in air and mounted in DPX. Counts of a total of two hundred spermatozoa per slide were made under a 4 mm. objective and × 8 oculars, using a blue filter and Beck 'Tenslite' illumination.

The cytological characteristics of cells killed by temperature shock were studied in suspensions made by diluting one drop of semen at 30° C. in eight drops of stain at 0° C.

Giemsa stain. Smears of whole semen were dried in air on a warm stage, fixed in neutral formal-saline (5 % formaldehyde) for 30 min. and then stained for $1\frac{1}{2}$ hr. in the following solution:

Giemsa (Gurr)	3 ml.
Sorensen phosphate buffer, pH 7·0	2 ml.
Glass-distilled water	35 ml.

Heidenhain's iron haematoxylin. Smears of whole semen could not be stained satisfactorily by this technique. They were therefore prepared from semen diluted in M/15-phosphate buffer (pH 7·4). These were dried in air, fixed in osmium tetroxide vapour for 10 min. at 37° C., washed overnight in running tap water, mordanted for 24 hr. in $2\frac{1}{2}$ % ferric alum solution, stained for a similar period in haematoxylin, differentiated in fresh alum solution and mounted in DPX.

Control suspensions were made by pipetting one drop of semen at 30° C. into eight drops of buffer solution at this temperature.

Suspensions of 'temperature-shocked' material were prepared by dilution with buffer at 0° C. This treatment was sufficient to kill all spermatozoa in the suspension.

Microscopical examination of unfixed material. Living semen was cooled slowly and handled at room temperature, after storage, if necessary, at 4° C. For study of the dead cell, spermatozoa were killed by pipetting 0·5 ml. semen at 30° C., drop by drop, into a 6 in. Pyrex test-tube pre-cooled to 0° C. in an ice-filled vacuum flask.

Samples of the whole semen were mounted under a cover-glass on a thick film of 1% agar in mammalian Ringer. Details of the technique will be published by Mr J. Smiles, National Institute of Medical Research, Mill Hill, London.

Direct observations were made with Beck phase contrast microscope equipment. Living and dead spermatozoa were photographed by direct and phase-contrast ultraviolet light. I am indebted to Mr Smiles for the photomicrographs which are reproduced in this paper.

Electron micrography. The spermatozoa were washed by slow centrifugation in phosphate buffer (pH 7·4), fixed in formal-saline for 10 min. and washed twice in distilled water. The grids were shadowed with gold-palladium (40–60%) at an angle of 20° to the horizontal.

Control material was cooled slowly to room temperature and handled subsequently at room temperature. 'Shocked' samples were obtained by pipetting 0·5 ml. whole semen at 30° C., drop by drop, into a 6 in. Pyrex test-tube pre-cooled in a freezing mixture of ice and salt to -10° C. and exposed to this temperature for 15 min. This treatment ensured death of all cells in any sample of whole semen. It was employed because the cytological characteristics of cells from semen 'temperature-shocked' in this manner could be definitely related to the structure of the dead cell. Because of the much larger sample sizes available for study by light microscopy, the significance of any morphological heterogeneity within samples containing both live and dead cells could readily be determined. This might not have been possible in samples of the size studied with the electron microscope. The electron micrography was carried out by Dr J. R. G. Bradfield, Cavendish Laboratory and Department of Zoology, University of Cambridge.

RESULTS

The appearance of the spermatozoa in films prepared by the nigrosin-eosin technique from freshly ejaculated active bull semen is shown in Pl. 17*a*.

The outline of the spermatozoon is clearly defined, and its bright refractile head is in sharp contrast to the dark background. The anterior end shows a bright distinct boundary zone, which becomes indistinct in the region of the equatorial axis. This appearance is closely comparable with that in the photomicrographs of unfixed living material (Pl. 18*a*, *c*) and in those of material fixed in the 'living' condition and stained with iron haematoxylin (Pl. 17*c*). The distinct boundary to the anterior pole of the sperm head marks the limits of the cap which, in Giemsa-stained preparations, is seen to invest this region of the head (Pl. 17*e*). The electron micrograph of fixed material illustrates this general appearance in greater detail and confirms the impression that the structure is closely applied to the sperm head, the outline of which is regular and uninterrupted (Pl. 19*a*). The posterior boundary of the cap is straight and bisects the head equatorially.

The appearance of dead spermatozoa in fixed and unfixed material is characteristically different from that of the living cell and of cells in which the living structure has been fixed.

In the nigrosin-eosin preparations, the 'dead' spermatozoa are stained with eosin and no longer contrast sharply with the background stain (Pl. 17*b*); the outline of the sperm head is indefinite and, in fact, the 'dead' spermatozoa in such preparations are readily distinguished from the 'living' ones without reference to their affinity for eosin. The cap is now inconspicuous; it shows an irregular outline and loosely envelops the anterior part of the sperm head.

This loosening of the attachments of the cap is more evident in the photomicrographs of dead unfixed spermatozoa. There is evidence of shrinkage along the posterior border of the cap which results in the appearance of an equatorial segment (Pl. 18*b*, *d*). The general pattern conforms with that in the photomicrographs of stained dead material (Pl. 17*d*). The profound nature of the structural change in the cap is clearly illustrated in the electron micrograph (Pl. 19*b*). These changes are also found in the dead spermatozoa in freshly ejaculated semen and in samples which have been stored for several days, but are more pronounced in the latter. The wide separation of the cap and sperm head which is frequently observed in such material appears to represent a later post-mortem change preceding complete detachment of the cap. These features are illustrated in Pl. 17*e*.

Table 1. *The relationship between the numbers of capless and dead spermatozoa*

Bull	No. of ejaculates studied	Average % dead cells	Average % capless cells
1	7	22·9	2·29
2	4	17·1	1·12
3	3	27·2	6·08
4	2	13·0	2·88
5	7	22·0	2·62
6	6	15·8	2·29
7	2	10·0	2·50
8	1	8·0	0·25
9	3	50·3	25·20
10	6	14·9	1·14
11	5	9·1	2·10
12	2	45·0	33·13

Table 1 summarizes some observations on the incidence of capless spermatozoa in freshly ejaculated semen. The proportion of capless cells was determined by counting 200 spermatozoa in each of two Giemsa-stained smears of whole semen.

The table shows that the proportion of capless cells is related to the proportion of dead cells. Analysis of the total data for individual ejaculates shows that this relationship is significant at the 0·1% level and provides clear evidence that denudation of the sperm head is associated with death of the cell.

DISCUSSION

Bull spermatozoa exhibit markedly altered morphological characteristics after death; any conclusions about the normal morphology of spermatozoa may, therefore, be open to serious criticism unless steps are taken to determine and control the viability of the material studied. In this way structural variations due to morbid changes in the cell may be recognized and their significance appreciated. The susceptibility of spermatozoa to lethal damage inflicted by sudden cooling suggests that particular care is required in the handling of semen prior to cytological study.

Of immediate interest are the changes which have been shown to occur in the sperm cap. In the living spermatozoon the cap is closely applied to the head, while in the dead spermatozoon it appears as a looser structure which may become detached as a fragile envelope. The changes in the structure of the cap which are seen after death are also responsible for the appearance of the equatorial segment which is not visible in the living spermatozoon. A survey of the published results of previous studies shows that there is still some doubt as to the identity and nature of the cap. An attempt will be made here to show that some of this confusion is traceable to the failure of previous observers to recognize the existence of post-mortem changes in its structure.

Blom (1945) confirmed the existence of a sperm cap in the bull and stallion and identified it as the galea capitis or *kopfkappe* previously described by Ballowitz (1891 *a*, *b*) and Retzius (1909). His review of earlier work suggests that he regards it as homologous with the structure described in other species as the *perforatorium* (Waldeyer, 1906), and *acrosome* (Bowen, 1924), to mention only two of the several names which exist. The present observations have shown that the sperm cap of the bull possesses a uniformly characteristic appearance in the living cell and confirm Blom's suggestion that detachment of the cap may be evidence of the death of the spermatozoon.

An examination of the literature which has followed Blom's paper suggests that while there is widespread acceptance of the existence of the galea capitis there is reluctance to identify it with the acrosome.

Rao & Hart (1948) noted the variations in the morphological appearance of the sperm cap which have been described above. They failed, however, to appreciate their significance. They suggest that in certain forms the acrosome can be seen within the cap. Their photomicrograph in support of this suggestion illustrates the features seen in Pl. 17*a*. The evidence for the existence of a second structure is unconvincing and does not justify the implication that the protoplasmic cap (galea capitis) is not the acrosome.

Bretschneider & van Iterson (1947) and Bretschneider (1949) have illustrated the galea capitis *in situ* as a fragile, loose envelope, and it is obviously identical with the cap dealt with in this paper. In all their illustrations, however, the characteristic features of the cap of the living cell have been lost. Bretschneider (1950) has recently brought forward evidence suggesting that the galea capitis originates as a double structure which sometimes persists as such and is encountered as a specific sperm defect associated with sterility in the bull (Teunissen, 1946; Hancock, 1949). He emphasizes, however, that he has been unable to demonstrate more than one cap-like structure in the mature spermatozoa of the normal bull, and his discussion of the results of comparative studies with the electron microscope of the spermatozoa of the guinea-pig, bee, squid and bull leave no doubt that he regards the galea capitis of the bull as the homologue of the acrosome of other species.

Randall & Friedlaender (1950), in a study of the ram spermatozoa with the electron microscope, claim to have demonstrated two cap-like structures. These are identified as the galea capitis and acrosome. The galea capitis is said to lie over the acrosome which only becomes visible after removal of the former; their separate illustrations of these two structures show that the acrosome corresponds closely in appearance with the cap of the bull spermatozoon in Pl. 19*a*; there is a similar correspondence between their illustration of the galea capitis and that of the cap of the bull spermatozoon in Pl. 19*b*.

Bishop (1951) has since claimed to have demonstrated in bull spermatozoa structures comparable with those reported for the ram by Randall & Friedlaender, although his illustrations furnish little evidence in support of this claim.

It is probably significant that in none of the papers cited above is there any evidence of an attempt to determine and preserve the viability of the spermatozoa in the semen studied. There can be little doubt that failure to distinguish between the structure of the cell before and after death has produced an erroneous picture of the normal appearance of the sperm cap. Equally there can be little doubt that the alleged existence of a galea capitis and an acrosome in the spermatozoon of the ram and bull is due to the failure to distinguish between the characteristic appearances of the cap before the death of the cell, and after. The structure which exhibits these characteristic changes is identical with the galea capitis described by Blom. In the absence of convincing evidence of other comparable structures it may be concluded that the terms acrosome and galea capitis have been used to describe the same structure.

It seems worth while to examine this conclusion in the light of other evidence provided by studies of spermatogenesis. Gatenby & Woodger (1921), in their study of spermatogenesis in the guinea-pig, attempted to resolve some of the confusion surrounding the identity of the cytoplasmic investment of the spermatozoon. It is noteworthy that the term galea capitis is absent from the list of terms tabulated by them in an attempt to systematize the nomenclature relating to this structure. It would seem probable that the term galea capitis has only been applied to the mature sperm. According to the above workers the *kopfkappe* is the equivalent of the structure described by Papanicolaou & Stockard (1918) as the *spermiocalyptrotheca* of the guinea-pig sperm. Gatenby & Woodger apparently did not identify any similar structure in their own studies of this species, but suggest as a simple alternative 'the covering membrane of the acrosome'. This structure was said by Papanicolaou & Stockard to invest the whole sperm head and cannot therefore be the homologue of the *kopfkappe* of Retzius. Gresson & Zlotnik (1948) have described the origin of the acrosome of the bull spermatozoon in terms which correspond with those used by Gatenby and his collaborators (Gatenby & Woodger, 1921; Gatenby & Beams, 1935) to describe its origin in other species, although the acrosome in the guinea-pig is exceptional in that it appears as a double structure. There is no doubt that the acrosome of the bull, described by Gresson & Zlotnik, is the cap dealt with in this paper and the structure which Blom described as the galea capitis. Thus the terms acrosome and galea capitis as applied to the bull spermatozoon are synonymous and it becomes necessary to reject one. The universal use of the term acrosome by cytologists in this country and the U.S.A. is sufficient justification for its retention.

SUMMARY

The microscopical features of living and dead bull spermatozoa are described. The findings are discussed in relation to previous observations on the structure of the head of the bull spermatozoon. The cytoplasmic cap of the living cell possesses a characteristic structure which is identical with that which has been ascribed to the acrosome. The altered structure of the cap of the dead spermatozoon is identical with that previously described as characteristic of a second cap-like structure, the galea capitis. It is concluded that the terms galea capitis and acrosome have been used to describe the same structure. It is suggested that the cytoplasmic cap of the bull spermatozoon should be called the acrosome.

I wish to thank Lord Rothschild, Department of Zoology, University of Cambridge, for his unremitting encouragement and criticism.

I am also indebted to Dr W. Jacobson, Strangeways Institute, Cambridge, for his generous guidance, to Dr A. Walton, Animal Research Station, Cambridge, for his help and interest, and to my colleague, Mr R. C. Campbell, for his statistical analysis of the data in Table 1.

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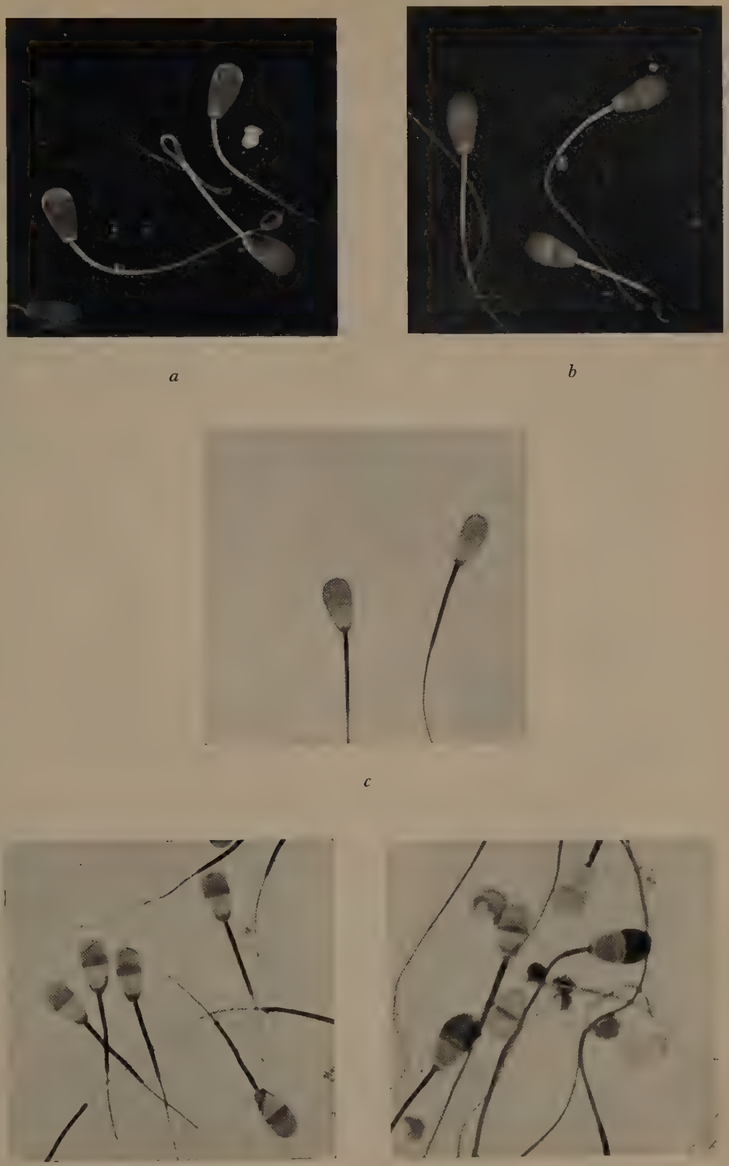
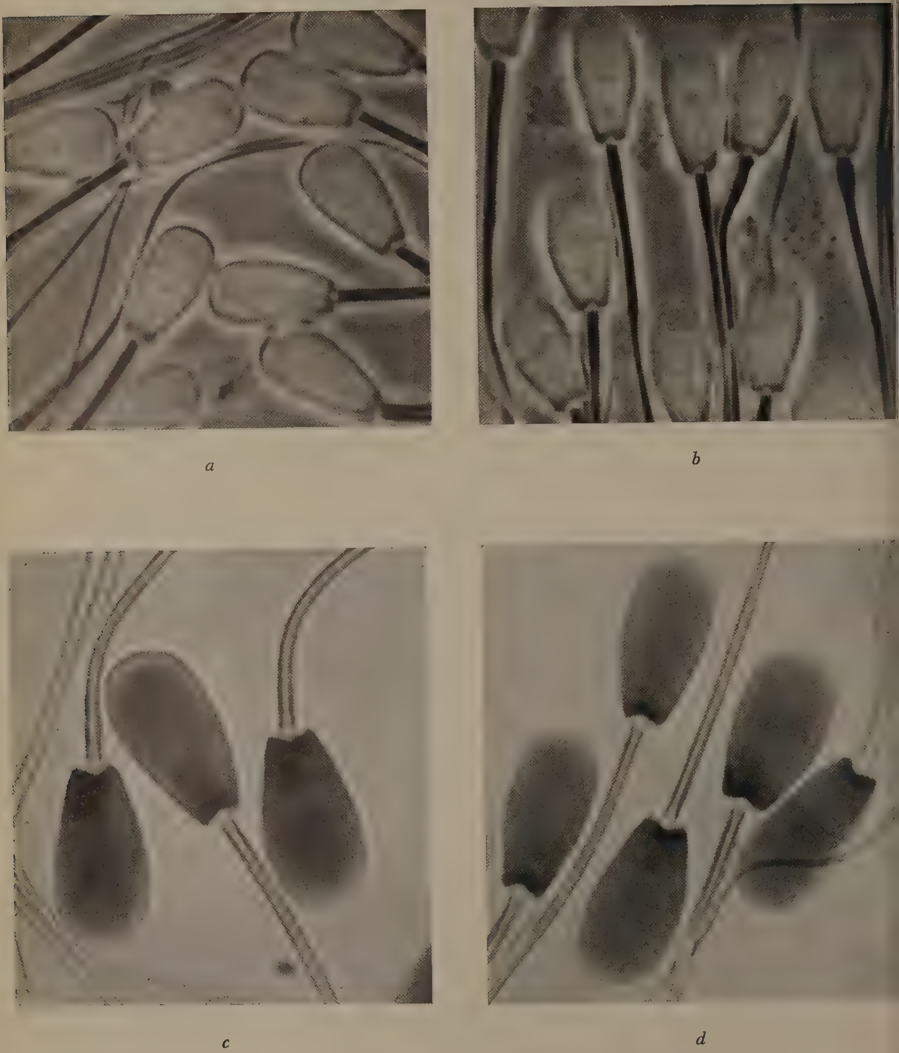


a *b*

c

d *e*

HANCOCK—THE MORPHOLOGY OF BULL SPERMATOZOA



HANCOCK—THE MORPHOLOGY OF BULL SPERMATOZOA

a

b

HANCOCK—THE MORPHOLOGY OF BULL SPERMATOZOA

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EXPLANATION OF PLATES

PLATE 17

a. Living spermatozoa. Nigrosin-eosin stain. × 950. *b.* Dead spermatozoa. Nigrosin-eosin stain. × 950. *c.* Living spermatozoa. Iron haematoxylin. × 950. *d.* Dead spermatozoa. Iron haematoxylin. × 950. *e.* Spermatozoa showing spontaneous detachment of the acrosome. Giemsa stain. × 950. Two spermatozoa show the acrosome *in situ*. A single free acrosome is shown adjacent to one of the four denuded heads (upper left).

PLATE 18

a. Living spermatozoa. Ultraviolet. 2750 A. Negative phase-contrast. × 2350. *b.* Dead spermatozoa. Ultraviolet. 2750 A. Negative phase-contrast. × 2350. *c.* Living spermatozoa. Ultraviolet. 2750 A. × 3300. *d.* Dead spermatozoa. Ultraviolet. 2750 A. × 3000.

PLATE 19

a. Living spermatozoon. Electron micrograph. Shadowed, gold-palladium. × 16,000. *b.* Dead spermatozoon. Electron micrograph. Shadowed, gold-palladium. × 16,000.

In Pls. 17 and 19, 'living' spermatozoa means spermatozoa in which the structure of the living cell has been preserved; 'dead' spermatozoa means spermatozoa in which the structure of the living cell has been destroyed.

THE FREEZING, DRYING AND STORAGE OF MAMMALIAN SKIN

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(*Received* 21 *January* 1952)

(With Three Text-figures)

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INTRODUCTION

It is now well established that mammalian skin and a variety of mammalian tumours will at least to some degree withstand being frozen to temperatures below the eutectic point of isotonic saline solution (Mider & Morton, 1939; Breedis, 1942; Snell & Cloudman, 1943; Webster, 1944; Briggs & Jund, 1944; Strumia & Hodge, 1945; etc.). The evidence for their survival is the fact that they may be successfully transplanted after thawing.

The object of the present investigation has been, first, to determine those conditions of freezing and thawing that result in the least possible damage to the various cellular and fibrous constituents of skin, and secondly, to determine the degree to which skin will withstand dehydration in the frozen state. Skin is a particularly suitable tissue for these experiments because of the ease with which grafts may be removed, subjected to various treatments *in vitro* and then grafted back orthotopically (i.e. to a position formerly occupied by skin) to the animal from which it was originally taken. The resistance of a tumour to freezing can be 'scored' only by its rate of growth after transplantation. A skin graft, by contrast, can be scored for its growth rate, its degree of pigmentation, the normality of its crop of hairs, the survival of sebaceous glands, the degree to which its fibrous organization remains intact, and so on.

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* British Empire Cancer Campaign Research Fellow.

A. THE FREEZING AND THAWING OF SKIN

Material and methods

All the experiments to be described have been carried out on the ear skin of adult rabbits weighing 2–3 kg. Animals bearing pigmented hair on their ears were selected wherever possible in order that melanoblast survival should be among the criteria used for scoring the grafts. As details of the operative procedures have been fully described elsewhere (Billingham & Medawar, 1951) they will be repeated here only in outline.

The grafts were cut from the shaved and lightly vaselined skin of the dorsum of the ear, each graft being a flat disk of skin about 0·25–0·5 mm. in thickness. Although thin, the grafts included the bases of most of the hair follicles, because the hairs emerge almost horizontally from the skin of the rabbit's ear. Elsewhere the hairs penetrate more deeply. Uniformity of size was achieved by defining the margin of each graft before cutting with a sharp cork borer of 9 mm. diameter. After being subjected to the various treatments *in vitro* to be described below, a standard test of viability was carried out by transplanting the grafts on to an extensive rectangular raw area prepared on the side of the same animal's chest (Billingham & Medawar, 1951, Pl. 5, figs. 4, 5). As a control a normal, untreated graft was always transplanted at the same time.

The grafts were first inspected on the eighth day after the operation, and thereafter at about 4-day intervals. Assessment of the degree to which the skin had resisted its treatment *in vitro* was based on the following criteria:

(*a*) Survival of the grafted epithelium, i.e. of the Malpighian cells, was demonstrated by the appearance of an annulus of migratory outgrowth from the original graft margin over the surrounding raw area and, of course, by the survival of the epithelium over the graft itself (Billingham & Medawar, 1951, Pl. 5, figs. 7, 8).

(*b*) The survival of the epidermal melanoblasts (pigmentary dendritic cells: Billingham, 1948, 1949) as shown by the regeneration of a crop of *pigmented* hairs and the presence of some pigmentation in the superficial epidermis itself. (There is independent evidence that epidermal melanoblasts are much less resistant than Malpighian cells to a variety of physical stresses. We have found that the soaking of skin grafts in distilled water may selectively destroy the majority of the melanoblasts, and Taylor (1949) demonstrated that the momentary application of a stick of solid carbon dioxide to the black hair-bearing skin of hooded rats caused permanent depigmentation.)

(*c*) The preservation of the fine fibrous organization of the dermis, as shown by the permanent conservation of an incisively distinct graft margin, the regeneration of a crop of hairs of normal density and orientation, and the preservation of the overall appearance that distinguishes ear skin sharply from the skin of the general integument. If the fibrous organization of the dermis of a skin graft has been seriously impaired, no more than a sparse hair crop is likely to regenerate, and the original graft centre, an epithelialized pad of dead-white and inert-looking dermal collagen, progressively diminishes in size and loses its identity.

Of the many possible rates at which skin could be frozen and subsequently thawed, two very widely different rates of freezing and of thawing were selected for investigation. Though these rates have not been accurately measured, the conditions under which they were obtained were strictly standardized and were therefore reproducible. The grafts were 'gummed out' flat, with their raw dermal surfaces outermost, on to the lightly vaselined surface of a narrow strip of copper foil 0·25 mm. thick, by means of which grafts could easily be manipulated. The application of vaseline to the skin before cutting the grafts helped them to stick to the copper.

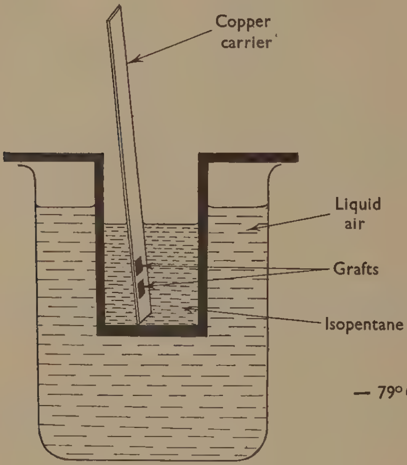

Fig. 1.

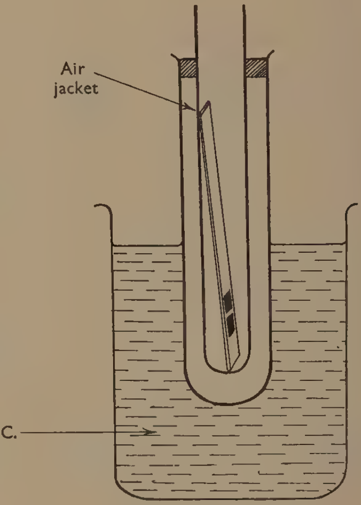

Fig. 2.

Fig. 1. Apparatus used for the procedure described in the text and Tables 1 and 2 as 'Quick freezing'. The grafts, lying flat on a thin copper carrier, are dropped into a copper vessel containing isopentane chilled to the temperature of liquid air.

Fig. 2. Apparatus used for the procedure described in the text and Tables 1 and 2 as 'Slow freezing'. The copper carrier with the grafts is lowered into a thick-walled air-jacketed test-tube which is immersed in an alcohol-dry ice mush at −79° C.

Quick freezing (Fig. 1) was achieved by plunging the copper carrier with the grafts as rapidly as possible below the surface of a bath of isopentane chilled in liquid air to a temperature below −150° C. (The isopentane is not itself toxic; Snell & Cloudman (1943) found that pieces of transplantable tumour tissue were perfectly viable after immersion in isopentane for half an hour at room temperature, and we have found it to be equally innocuous to skin.)

Slow freezing (Fig. 2) was carried out by placing the copper strip bearing the grafts into the inner of two thick-walled co-axially assembled test-tubes, the outer tube being separated from the inner by an air gap of 3 mm. This assembly was first immersed for 15 min. in a bath containing an ethyl alcohol/solid carbon dioxide freezing mixture at −79° C. and then transferred to a bath of liquid air for a further 15 min. Finally, to ensure that the tissue had in fact been frozen to a temperature below −150° C., the copper strip was withdrawn and plunged directly into isopentane chilled in liquid air.

Quick thawing was achieved simply by plunging the metal graft-carrier bearing the frozen grafts directly into about 50 ml. of Ringer's solution warmed to 37° C.

Slow thawing was obtained when the copper strip bearing the grafts was dropped into an empty glass vessel surrounded by crushed ice. The layer of frost which formed on the metal strip when it was exposed to the air did not thaw for at least 5 min. in this cold chamber.

In Tables 1 and 2 we use the terms 'quick freezing', 'slow thawing', etc., in the sense of these definitions.

Pre-treatment of the grafts. Before being subjected to one or other of the freezing procedures described above the grafts were soaked for 1 hr. at room temperature in either Ringer's solution or in a 15% w/w solution of glycerol in Ringer's solution. In using glycerol we followed the lead of Polge, Smith & Parkes (1949), who found that it protected fowl spermatozoa from the otherwise fatal effects of vitrification or 'snap freezing'.

The survival of intact skin after freezing and thawing

The epithelium of the rabbit's ear-skin grafts used in these experiments was found to be extremely tolerant to variation in the rate at which it was frozen. Grafts of Ringer-treated and glycerol-treated skin were found to withstand both the quick-freezing and the slow-freezing procedures. With respect to the rate at which it was thawed, however, the skin proved more exacting. Grafts that had been soaked in Ringer's solution before freezing survived quick thawing but invariably succumbed to slow thawing. Pre-treatment with glycerol allowed the grafts to withstand slow thawing. The results are summarized by Table 1. Evidently the glycerol caused the tissue to freeze and/or thaw in such a way that the cells were protected from the otherwise fatal consequences of slow thawing.

Table 1. *Showing the survival* (+) *or non-survival* (o) *of rabbit ear-skin epithelium under various conditions of pre-treatment, freezing and thawing*

Rate of freezing	Rate of thawing	Pre-treatment	
		Ringer	15% glycerol
Quick	Quick	+	+
Quick	Slow	o	+
Slow	Quick	+	+

The scoring of the results by the mere survival or non-survival of the epithelium of the grafts does not allow the various methods of freezing and thawing to be arranged in order of merit. When the other criteria by which the well-being of grafts may be assessed were taken into consideration, slow freezing was invariably found to be preferable to quick freezing. This confirms the results obtained by previous workers on both tumour tissue and skin. The superiority of slow freezing is made particularly clear when the treatments are classified by their effects on epidermal melanoblasts. Table 2 shows that the melanoblasts of rabbit's ear-skin grafts pre-treated with Ringer's solution survive neither quick freezing nor *slow* thawing; slow freezing is essential. Pre-treatment of the grafts with 15% glycerol in Ringer's solution protected the melanoblasts against the destructive effects of quick freezing but not of slow thawing. The actual concentration of glycerol required to give this effect proved not to be critical. Concentrations as high as 30% gave satisfactory results, and so did concentrations below 15%.

Table 2. *Showing the survival* (+) *or non-survival* (o) *of the melanoblasts of rabbit's ear skin under various conditions of pre-treatment, freezing and thawing*

Rate of freezing	Rate of thawing	Pre-treatment	
		Ringer	15% glycerol
Quick	Quick	o	+
Quick	Slow	o	o
Slow	Quick	+	+

Freezing and thawing of suspensions of epidermal cells

In an attempt to demonstrate even more decisively the protective action of glycerol pre-treatment, freezing and thawing, experiments were carried out not on organized skin grafts but on suspensions of isolated epidermal cells, including both melanoblasts and Malpighian cells. Our belief that isolated cells would prove more susceptible to freezing and thawing was strengthened by the reports of earlier workers that saline suspensions of tumour cells were more readily inactivated than tumour tissue frozen *en masse* (Mider & Morton, 1939). The fact that leucocytes do not withstand freezing may also be relevant (Jacoby, personal communication of results which we have confirmed). The technique for the preparation of viable suspensions of epidermal cells has been fully described elsewhere (Billingham & Medawar, 1951). Slight modifications of the standard freezing and thawing procedures were necessary to handle these cells in fluid suspension.

Quick freezing was carried out by allowing the cell suspension (contained in about 0·5–1 ml. of fluid) to fall in small drops down the side of a thin-walled glass tube immersed at an angle and slowly rotated in liquid air. Freezing was almost instantaneous.

Slow freezing was achieved by placing about 0·5–1 ml. of the cell suspension in a thin-walled glass test-tube fitted with an air jacket which was then immersed in liquid air. Freezing began after about 5 min. When it was complete the air jacket was removed and the tube bearing the frozen material was immersed directly in liquid air for 5 min.

Rapid thawing was brought about simply by immersing the tube bearing the cells in a water-bath at 37° C. Slow thawing was not attempted.

The test of viability was the same in principle as that used for orthodox grafts. After thawing the cell suspension was diluted with Ringer's solution and the cells spun down in the centrifuge. The clear supernatant fluid was discarded and the now compact mass of cells taken up with a pipette and transplanted, as if it were an organized graft, to the centre of the usual raw area. The regeneration of a sheet of epithelium was ample evidence that at least some of the cells had survived. The fate of the melanoblasts was indicated by the presence or absence of streaks of pigmentation in this sheet of epithelium.

Table 3. *Showing the survival* (+) *or non-survival* (o) *of suspensions of separated ear skin epithelial cells after quick and slow freezing preceded by Ringer or glycerol treatment*

Rate of freezing	Rate of thawing	Pre-treatment	
		Ringer	15 % glycerol
Quick	Quick	o	+
Slow	Quick	o	+

Results. Cells suspended in Ringer's solution did not withstand even slow freezing followed by quick thawing—the combination that had proved best for intact skin. Both Malpighian cells and melanoblasts survived both quick and slow freezing after they had been suspended in Ringer's solution containing 15 % glycerol. The results are set out in Table 3.

The storage of skin in the frozen state

The prolonged storage of skin grafts has long been recognized to be a clinical problem of some importance, and a reliable method for storing tissues without impairment of their vitality is desirable for a variety of experimental purposes. The literature on the storage of skin at temperatures below 0° C. reveals inconsistencies. For example, Baxter & Entin (1948) report that human skin deteriorates rapidly upon storage below 0° C., but Strumia & Hodge (1945) report that it survives without deterioration for *at least* two months.

A set of four standard grafts was removed from the dorsal ear skin of each of a panel of rabbits. Two of each quartet of grafts were pre-treated for 1 hr. with Ringer's solution, and the other two with 15 % glycerol in Ringer's solution. After blotting off excess fluid the grafts were dropped into small, empty, stoppered glass tubes and placed in a metal box for protection. The box was then stored in contact with solid carbon dioxide at -79° C. in a 5 gal. Dewar flask. (Under these conditions the grafts were slowly frozen.)

Pairs of tubes bearing the stored grafts were withdrawn from time to time and rapidly thawed by immersing the tubes in a water-bath at 37° C., while simultaneously pouring in warm Ringer's solution. The standard transplantation test for survival was then carried out.

Results. The longest storage period we have so far used is 4 months. In no case was there any outwardly perceptible difference between stored grafts that had been pre-treated with glycerol solution and freshly removed grafts that had been transplanted at the same time as controls. The grafts pre-treated with Ringer's solution were, however, always inferior; they regenerated a sparser hair crop showing varying degrees of depigmentation, and the grafts, which shrank and tended to lose their identity, never completely recovered the appearance characteristic of ear skin.

These results show that skin grafts which have been pre-treated with glycerol solutions can be stored without apparent deterioration for at least 4 months at −79° C. We see no reason to doubt that they would have survived storage under the conditions described for periods greatly exceeding even the most optimistic expectation of life of their donor. Although grafts pre-treated with Ringer's solution were invariably inferior there is no evidence that their inferiority increased with the duration of storage.

B. THE VIABILITY OF SKIN AFTER DEHYDRATION FROM THE FROZEN STATE

Earlier work

Gye (1949) and Gye, Begg, Mann & Craigie (1949) have shown that frozen and dried preparations of a number of mouse tumours will give rise to tumours of the same character after rehydration and implantation into hosts of suitable genetic constitution. Although other constructions have been put upon this evidence, it establishes a *prima facie* case for supposing that at least some mammalian cells can withstand a high degree of dehydration. Passey & Dmochowski (1950), Passey, Dmochowski & Lasnitzki (1950) and Dmochowski & Millard (1950) have now shown that this is indeed the case, for such desiccates may be proved to contain living cells by cultivation *in vitro*. Unfortunately, none of these authors provides quantitative estimates of the degree of dehydration that was achieved, and there is no evidence that any mammalian tissue can withstand dehydration to, say, a residual water content of as little as 5%. We made a number of preliminary attempts to dehydrate skin grafts by methods closely similar to those described by Gye and his colleagues (cf. Craigie, 1949). Transplantation tests failed to reveal any degree of survival, even when the grafts had been impregnated before freezing and drying with plasma or serum, or isotonic solutions of dextrose, maltose and dextran (a bacterial polysaccharide). Since this failure was presumably the outcome of achieving too high a degree of dehydration, we set ourselves to find out what degree of dehydration a skin graft can in fact withstand.

Methods

General considerations. Since any computation of the amount of moisture remaining in a graft after freezing and drying entails the complete desiccation, or at all events the destruction of the graft, it was clearly impossible to do moisture determinations and tests for viability on one and the same piece of skin. All our experiments were therefore done accurately in duplicate. Two sets of grafts were removed from the ear and simultaneously frozen and dried in separate vessels on the same apparatus. All the appropriate weighings were done with one set of grafts, and the transplantation test of viability was done upon the other. The grafts were frozen and dried either directly, i.e. without special treatment, or after impregnation for an hour with various concentrations of glycerol in Ringer's solution. The procedure that will be described hereafter is that which relates to the handling of glycerol-impregnated grafts; it may be taken to apply to the handling of untreated grafts simply by the omission of the procedures that relate to glycerol treatment.

The weight determinations made upon the weighed set of grafts ended with their complete dehydration. Knowing the original weight of the grafts and their final dry weight after allowance had been made for the weight of glycerol and Ringer salts added during their preliminary impregnation, it was possible to compute the water content of the original grafts. As a check upon this *indirect* estimate and as a control against unsuspected sources of error, we invariably removed a third set of grafts from neighbouring areas on the rabbit's ear, and, having weighed them, desiccated them directly without further ado. These *direct* measurements of the initial water content of the grafts are those entered in brackets in Table 6 immediately after the indirect estimates. The correspondence between the two sets of figures is good. The difference between them is due in part to errors of measurement and in part to real differences between the initial water content of neighbouring pieces of skin.

Detailed procedure. The dorsal surface of a rabbit's ear was cleaned and shaved and the cuticle greased with the thinnest possible film of vaseline. A number of rectangular strips, each approximately 3 × 1 cm. in area, were then removed as Thiersch grafts (cf. Billingham & Medawar, 1951) as thinly as it is possible to cut them. The ear was held under tension during the cutting of the grafts, and the grafts were only rarely contaminated with blood.

The strips intended for weight determinations (together rarely more than 100 mg. wet weight) were blotted to remove exuded lymph and at once dropped into a weighed 50 ml. stoppered conical Erlenmeyer flask which had been stored overnight in a vacuum desiccator over P_2O_5 awaiting use; the bottom of the flask had been lined beforehand with a thin film of high vacuum grease. The flask was weighed; its stopper was then removed and the grafts individually flattened out and caused to adhere closely to the bottom of the flask with their raw sides facing upwards. The slight loss of moisture accompanying this operation was determined by a second weighing. A film of a glycerol solution in Ringer of known composition was then applied to the dermal surface of each graft; the flask was thereupon weighed and allowed to stand for 1 hr.

The grafts intended for the viability test were treated in the same way but for the use of sterile apparatus and the omission of weighing.

After an hour's impregnation both sets of grafts were frozen side-by-side by immersing the two flasks in a bath of alcohol slowly cooled from room temperature to below that at which dehydration was to take place by the periodic addition of chips of 'dry ice'.

The two flasks were then coupled to the apparatus illustrated in Fig. 3, consisting of a two-stage vacuum-pump with a P_2O_5 and a -79° C. (dry-ice/alcohol) vapour trap; dehydration was conducted at a pressure between 0·005 and 0·001 mm. Hg and at a temperature held between -22 and -25° C. for glycerol-treated grafts or between -30 and -32° C. for untreated grafts. (The lower temperature was adopted simply to slow down the rate of dehydration in the absence of glycerol.) Dehydration continued from 1 to 12 hr., according to the degree which it was hoped to achieve.

When dehydration had gone on for as long as was intended, air was very slowly admitted into the apparatus so as to reach the flasks after passage through the two vapour traps. The flask containing the grafts for the viability test was then uncoupled and instantly filled with Pannet and Compton's solution (essentially a phosphate-buffered Ringer) at body heat. Rehydration and thawing were thus brought about simultaneously and as quickly as possible; the grafting operation to test their viability was done 1–2 hr. later.

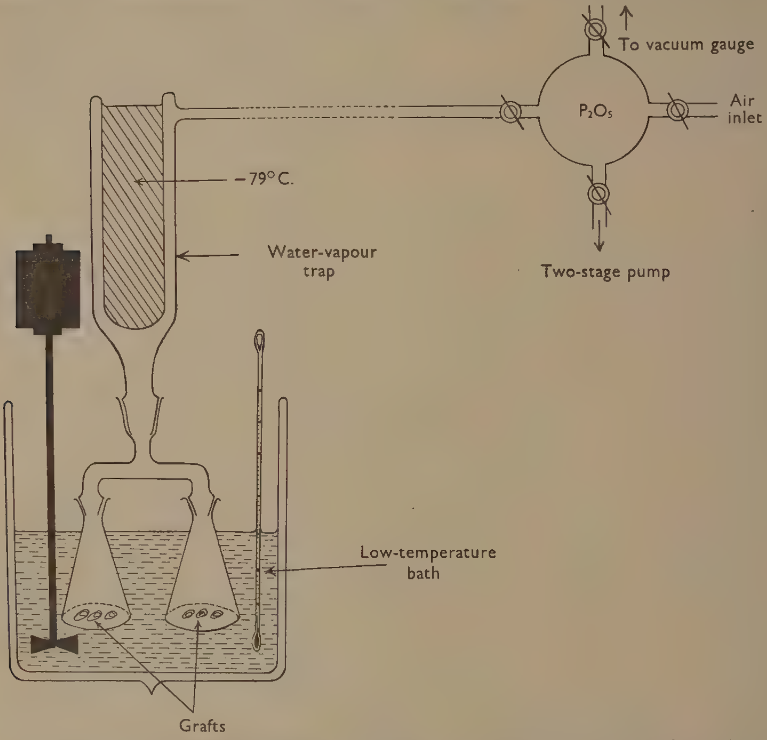

Fig. 3. Illustrating the apparatus used for drying skin in the frozen state. Skin grafts are laid flat on the bottom of two 50 ml. Erlenmeyer flasks with their dermal (inner) sides upwards; the grafts in one flask are used for weighings and in the other for viability tests. The flasks are coupled to a two-stage Speedivac pump via two vapour traps, one of alcohol-dry ice mush and the other of phosphorus pentoxide. Air may be so admitted at the end of the experiment as to reach the flasks only through the two vapour traps. During the course of the experiment the flasks are kept within a constant range of temperature by means of a stirred spirit bath to which chips of dry ice are added from time to time.

The flask to be weighed was raised from the cooling bath and allowed to reach room temperature in its own time. After uncoupling, traces of high vacuum grease were removed from the neck with a gauze swab damped with ligroin and the flask was restoppered and weighed. The residual moisture was then estimated by dehydration to a constant weight (in practice for 18–24 hr.) at 50° C. over P_2O_5 in a vacuum desiccator under reduced pressure. (Glycerol is *not* lost under these conditions.) A final weighing completed the experiments.

A representative calculation is set out in Table 4.

Table 4. *A representative calculation (relating to the first entry in Table* 6) *illustrating the procedure adopted in determining the resistance of skin grafts to a dehydration of known degree in the presence of glycerol solution*

(Note the good correspondence between the directly and indirectly computed estimates of the percentage of water in the original grafts. For full description of the procedure, see text. All weights are expressed in grams.)

Indirect drying

A = prepared Erlenmeyer flask	38·1986
$B=A+$grafts	38·3230
$C=B$ after flattening out grafts	38·3218
$D=C+$glycerol-Ringer film	38·5106
$E=D$ after freeze-drying for 1 hr.	38·2682
$F=E$ after complete desiccation	38·2456
Graft weight $(B-A)$	0·1244
Loss of vapour due to flattening $(B-C)$	0·0012
Weight of glycerol-Ringer film $(D-C)$	0·1888
Glycerol (3·767 %) therefore	0·0071
Ringer's solution 0·1888–0·0071	0·1817
Ringer salts, 1 %	0·0018
Initial weight of system 0·1244+0·1888	0·3132
Initial water in system $(D-F+0{\cdot}0012)$	0·2662
Final residue $(F-A)$	0·0470
Graft dry weight (0·0470–0·0071–0·0018)	0·0381
Initial graft water (0·1244–0·0381)	0·0863
Weight of system after freeze-drying $(E-A)$	0·0696
Residual water after freeze-drying $(E-F)$	0·0226
Initial graft moisture, $100\dfrac{0{\cdot}0863}{0{\cdot}1244}$	69·4 %
Initial water in system, $100\dfrac{0{\cdot}2662}{0{\cdot}3132}$	85·0 %
Water after freeze-drying, $100\dfrac{0{\cdot}0226}{0{\cdot}0696}$	32·5 %
Percentage dehydration, $100\dfrac{0{\cdot}2662-0{\cdot}0226}{0{\cdot}2662}$	91·5 %
Glycerol conc. after freeze-drying (as aqueous solution), $100\dfrac{0{\cdot}0071}{0{\cdot}0071+0{\cdot}0226}$	23·9 %

Survival test: Positive

Direct drying

A = weighing bottle	8·8351
$B=A+$grafts	8·9078
$C=B$ after complete desiccation	8·8582

$$\text{Graft moisture}=100\,\frac{B-C}{B-A}=68{\cdot}2\,\%$$

Results

Untreated grafts (Table 5). The entries in the last column of Table 5 show that none of our untreated grafts has survived dehydration to a degree below that represented by a final overall water content of 25 %. (Grafts still containing as much as 25% of residual water look and feel bone dry; their outward appearance is therefore most misleading.) The pattern of distribution of the residual water in the grafts is, unfortunately, unknown. It certainly cannot be assumed to be homogeneous.

Glycerol-impregnated grafts. The distribution of residual water in grafts dried after impregnation with glycerol is likely to be much more homogeneous; the final overall glycerol concentration rises to between 24 and 79% (Table 6, penultimate column), the grafts become optically clear and stiff instead of pliable, and the hygroscopic properties of glycerol make it reasonably likely that the distribution of intercellular water is not grossly uneven. Moreover, unless cell surfaces are freely permeable to glycerol, water cannot fail to have been osmotically withdrawn from within the cells during the later stages of dehydration, for glycerol-water systems are fluid at −22° C. at glycerol concentrations ranging from about 50 to 80%.

Table 5. *Showing the survival* (+) *or non-survival* (o) *of skin grafts which have had no special preliminary treatment, after dehydration from the frozen state to the degree shown in the second column*

Initial graft H_2O (%)	Final H_2O (%) after dehydration	Survival
70	46	+
72	43	+
72	42	+
67	34	+
71	29	+
69	25	+
71	23	o
71	17	o
67	11	o

Table 6 shows that we have obtained survival of glycerol-impregnated skin grafts after dehydration resulting in not less than 24% overall residual water content. Evidently treatment with glycerol does not increase the resistance of skin to dehydration. The residual water content is not, however, the only variable; for as dehydration proceeds, the concentration of glycerol rises to what might well be a toxic level. For this reason we varied the initial concentrations of glycerol used for soaking the grafts (Table 6, column 2), and it will be seen from Table 6 that one graft survived dehydration to 24% residual water with a final glycerol content of 52%, although another graft failed to survive dehydration to 13% residual water when the final concentration of glycerol was only 45%.

Non-toxicity of glycerol. To make quite certain that glycerol is not toxic over the range of concentrations and lengths of exposure adopted in our experiments, freshly removed skin grafts were floated on or immersed in glycerol solutions of high concentrations with continual stirring. Such grafts become optically clear and as stiff as cardboard of comparable thickness. They were rehydrated by immersion in Ringer's solution *without* stirring and then transplanted back to their donors to test their viability. The epidermis of skin grafts so treated has been shown to survive exposure to 'absolute' (98·1%) glycerol for 8 hr. at 0° C. and to 80% glycerol for

2 hr. at room temperature, and all the grafts regenerated a sparse crop of new hairs. No attempt was made to prolong the time of exposure at these concentrations to determine the cell death points, and the grafts might well have survived much longer treatments.

Suspensions of isolated epidermal cells were understandably more vulnerable. Cell suspensions spun down from citrated saline and resuspended with continual stirring in 80 % and in 98·1 % glycerol at room temperature survived exposure for 35 min. but not for 1 hr. It may at all events be said without reserve that the toxicity of glycerol may be completely disregarded in interpreting our experiments on freezing and drying. Indeed, it may be that the dehydration of tissue by passage through glycerol solutions of ascending concentrations, much as the histologist dehydrates his tissues with ethyl alcohol, is a better method of determining their resistance to desiccation than drying in the frozen state.

Table 6. *Showing the survival* (+) *or non-survival* (o) *of skin grafts soaked in a glycerol-Ringer solution of the concentration shown in column* 2 *and then dried in the frozen state to the degree shown in column* 4

(The figures in brackets following the entries in column 1 are the moisture contents of grafts cut from nearby donor areas, estimated by direct dehydration. The penultimate column expresses the final concentration of glycerol as an aqueous solution in the residual water.
The data relating to the experiment entered in the first row are set out in full in Table 4.)

Initial graft H_2O (%)	Glycerol (%) (pre-treatment)	% water in system		Final (%) glycerol	Survival
		Initial	Final		
69 (68)	3·77	85	33	24	+
71 (71)	12·79	79	30	41	+
71 (69)	12·79	79	24	52	+
71 (71)	14·77	80	20	67	o
74 (70)	14·77	82	18	73	o
69 (68)	14·77	79	18	68	o
68 (65)	7·48	84	17	59	o
70 (70)	14·77	80	16	71	o
65 (65)	14·77	78	15	71	o
72 (73)	12·79	79	13	65	o
65 (63)	3·77	83	13	45	o
75 (75)	12·79	82	13	76	o
70 (68)	14·77	79	12	79	o

GENERAL DISCUSSION

Our results confirm and amplify the experience of a number of other investigators (see above) in showing (*a*) that skin withstands freezing to the temperature of liquid air; (*b*) that it is very tolerant to variations in the rate of freezing; (*c*) that on all scores slow freezing is preferable to rapid freezing—indeed, epidermal melanoblasts will not survive quick freezing unless protected by glycerol solutions; (*d*) that slow thawing is harmful; and (*e*) that, as Polge *et al.* (1949) have shown to be true of spermatozoa, glycerol solutions protect the more vulnerable elements of skin against the harmful effects of either quick freezing or of slow thawing, or of both. We also find (*f*) that skin may be stored for long periods at −79° C. without appreciable deterioration.

A few of these observations deserve comment. It has been the general experience of those who use continued growth and cell division as a criterion of survival that tissue is better preserved by slow freezing than by fast. On the other hand, it has been insistently argued by other workers, notably by Dr Luyet and his colleagues (Luyet & Hodapp, 1938; Luyet & Hartung, 1941*a*, *b*), that ultra-rapid freezing leading to 'vitrification' is theoretically to be preferred. The hypothesis upon which this preference is based is that rapid freezing prevents the formation of ice crystals in the cooling system, which solidifies in a virtually amorphous state, and so avoids the mechanical damage that they may cause. Unfortunately, this hypothesis leads to predictions that are not empirically fulfilled. It follows either that the hypothesis is wrong or that it has been mistakenly applied.

The storage of skin at very low temperatures under conditions (pre-treatment with glycerol solution and slow freezing) that render it no less capable of growth and differentiation than a freshly removed graft has both a clinical and an experimental bearing. Clinically, it makes possible the institution of efficient 'skin banks' for grafting purposes. Whether or not it is desirable or useful to have such banks depends upon many considerations that need not be discussed here. One of them is whether the administration of cortisone to human beings prolongs the life of skin grafted from one individual to another, as it is known to do so in rabbits (Billingham, Krohn & Medawar, 1951*a*, *b*). Our principal object in studying the storage of skin was to make use of it for experimental purposes. For example, one possible approach to the problem of the causes of senescence is to graft tissue from a young animal to its own self when it has grown older. Such an age chimaera (an organism whose parts are of the same genetical constitution but of different developmental ages) can be realized by the appropriate use of storage methods.

The manner in which glycerol exerts its beneficial effects is unknown. Presumably it achieves some degree of initial dehydration; it lowers the freezing-point of water down to a maximum of about −40° C. at a concentration of about 70 %; it certainly changes the 'grain' of the frozen solid in a conspicuous way; and its viscosity will be such as to promote a high degree of supercooling. Any of these physical properties may be important.

Our results show that untreated skin and skin impregnated with glycerol solutions do not survive a degree of dehydration that results in a final overall water content of less than about 25 %. It seems hardly necessary to pursue the problem of storing skin in the dry state, since adequate methods have now been devised for storing it by freezing alone. We wish to call attention to the necessity for supporting any claim that a tissue withstands 'desiccation' by factual records of its final water content. Some authors, admittedly as a rough guide, have given estimates of the 'percentage dehydration' of the partially dried system (see Table 4). Such estimates cannot but be highly misleading. In our experience, for example, 92 % of the initial water in the system 'impregnated skin + film of glycerol solution' had to be removed to achieve a final water content of 24 % (Table 6, third row entry); but this 'percentage dehydration' could be quite arbitrarily increased merely by adding more water to the initial system, a procedure that need not affect the final water content.

SUMMARY

1. The viability and general preservation of skin that has been subjected *in vitro* to freezing, drying and other procedures may be tested by transplanting it back to the animal from which it was originally removed.

2. Rabbit's ear skin withstands either slow or rapid freezing down to the temperature of liquid air.

3. Slow freezing gives better preservation than rapid freezing, but impregnation with glycerol solutions protects skin against some of the harmful consequences of rapid freezing. Epidermal melanoblasts do not withstand rapid freezing unless they have been protected by glycerol solutions.

4. Rapid thawing gives better preservation than slow thawing. Malpighian cells of the epidermis survive slow thawing after impregnation with glycerol solutions but not after impregnation with Ringer's solution.

5. Skin slowly frozen after impregnation with glycerol solution and stored for 4 months at −79° C. was indistinguishable, on transplantation, from a freshly removed graft. The storage of skin at −79° C. does not result in progressive deterioration during the period of storage.

6. It is shown that the toxicity of glycerol to the various cellular constituents of skin is so slight that it may be disregarded. Isolated epidermal cells withstand exposure to 98·1 % glycerol at room temperature for half an hour.

7. Skin does not withstand dehydration to a degree that results in a final overall water content below about 25 %. Impregnation with glycerol solutions does not increase its capacity to withstand dehydration.

This work has been supported by expenses grants from the British Empire Cancer Campaign and the Department of Plastic Surgery, University of Oxford. Miss Jean Morpeth has given invaluable technical assistance throughout.

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THE FERTILIZATION REACTION IN THE SEA-URCHIN

THE BLOCK TO POLYSPERMY

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(*Received* 23 *January* 1952)

(With Six Text-figures)

INTRODUCTION

The experiments described in this paper are concerned with the reactions of sea-urchin eggs to high concentrations of spermatozoa of the same species. Several papers on this and allied subjects have recently been published, and it may therefore be convenient to summarize the results so far obtained. The first series of observations involved the measurement of the time taken for a visible change in the cortical structure of the egg, which occurs at fertilization, to pass completely over the egg surface (Rothschild & Swann, 1949). It has of course been known since the nineteenth century that the surface of the sea-urchin egg changes at fertilization, and the method of observing this change, whether with polarized light, dark-ground illumination or ordinary light, all of which have been used at one time or another, is of no great interest in this context. The new feature in our work was to measure the conduction velocity of this change. The total conduction time was about 20 sec. at 18° C. As no faster post-insemination change had been observed, it was natural to consider whether this one could be the block to polyspermy. There were two methods of trying to answer this question. The first was to investigate the effect of polyspermy-inducers, such as nicotine, on the conduction time of the cortical change. If this change were the block to polyspermy, polyspermy-inducers might be expected to make the conduction time longer, unless they acted by increasing the chances of a successful sperm-egg union. Nicotine had no effect on the conduction time of the cortical change (Rothschild & Swann, 1950); but a consideration of its alternative mode of action led to the second method of investigation, an examination of the probability of a collision between a spermatozoon and an egg being successful (Rothschild & Swann, 1951*b*). If we think in terms of sperm-egg *collisions* and the probability of their being successful, an assumption must be made about the way such collisions take place; it is that suspensions of eggs and spermatozoa can be treated as spheres in assemblages of gas molecules, in which case an estimate of the probability of a successful collision can be made. The concept of the sperm-egg collision is not essential for a quantitative description of what happens when eggs and spermatozoa are mixed together. The relationship between the number of eggs fertilized and the time of contact between the eggs and the spermatozoa can be described in terms of a fertilization parameter α (Rothschild, 1952), the value of which depends on the condition of the eggs and spermatozoa, the density of the latter and the speeds at which they swim. This fertilization parameter does not involve any assumptions about sperm-egg collisions or the probability of their being successful, though it can be described in terms of these two variables.

These experiments depended on developing a technique whereby eggs and spermatozoa could be brought into contact with each other for known periods of time and then functionally separated. This technique suggested a method of investigating the conduction time of the block to polyspermy. Eggs were placed in contact with spermatozoa for a known time, 25 sec., at the end of which the spermatozoa were killed; later, the number of monospermic, polyspermic and unfertilized eggs were counted. Simultaneously, an identical experiment was done, but instead of killing the spermatozoa at the end of the 25 sec., more were added. This increased the number of polyspermic eggs, showing that during the original sperm-egg interaction time, all the blocks to polyspermy were not completed; from which it was concluded that the conduction time of the block to polyspermy was relatively long, being of the order of ten or more seconds (Rothschild & Swann, 1951*a*). These results were of a preliminary nature, and in a later paper (Rothschild & Swann, 1951*b*) we said they would be systematically repeated. At the same time, certain difficulties in there being a low-speed block to polyspermy but a comparatively small incidence of polyspermy at normal sperm densities were discussed in detail.

Since that time, a simpler method of investigating the conduction time of the block to polyspermy in the sea-urchin egg has been devised, which is the subject of this paper.

MATERIAL

Eggs, with their jelly-coats intact, and spermatozoa of *Psammechinus miliaris* were used. The room temperature varied from 16 to 18° C. during the season.

EXPERIMENTAL PROCEDURE

The experimental procedure depended on the following self-evident thesis. Suppose, for example only, that a number of eggs are all fertilized at $t=0$ and that the block to polyspermy is complete at $t=5$ sec. After 5 sec. there will be a certain number of polyspermic eggs in the egg population. The eggs will not all be polyspermic because some of them will not have sustained more than one successful sperm-egg collision during that 5 sec. As, however, all the blocks to polyspermy are complete by 5 sec., the number of polyspermic eggs will never be greater than it is at 5 sec. If the proportion of polyspermic eggs is 0·5 after 5 sec., the proportion must be 0·5 after 6, 10, 30, or 180 sec. It follows from this argument that if we take a series of egg suspensions and fertilize all the eggs in them at $t=0$, and if we 'remove' the spermatozoa from these egg suspensions at various times after $t=0$, say, 5, 10, 15 and 40 sec., then the time after which there is no increase in the incidence of polyspermy will be the conduction time of the block to polyspermy. Conversely, any decline in the incidence of polyspermy at $t=r$, as compared with the incidence of polyspermy at $t=s$, where $r>s$, will be due to sampling errors or mistakes in deciding whether an egg is polyspermic or monospermic. The first problem to be resolved in an experiment of this type is how to fertilize all the eggs at $t=0$. In a previous paper (Rothschild & Swann, 1951*b*) it was shown that when eggs and spermatozoa are mixed together and the number of spermatozoa per ml. is $3\cdot67\times10^5$, some 12 sec. must elapse before half the eggs in the suspension are fertilized. The incidence of polyspermy at such sperm densities is too low for accurate calculations based on that incidence. If the sperm density in contact with the eggs is increased by a factor of 300, however, both difficulties are resolved, for a significant number of eggs become polyspermic and all eggs in the suspension are fertilized, for the first time, in the neighbourhood of $t=0$.

The experimental method requires first, that the eggs and spermatozoa be rapidly mixed together, and secondly, that after they have been in contact with each other for a particular time, the reaction should be suddenly stopped. This was achieved by adding 20 ml. of sperm suspension to 2 ml. of egg suspension and, after the required sperm-egg interaction time, transferring the 22 ml. of eggs and spermatozoa into 1000 ml. of sea water. This reduces the density of the spermatozoa in contact with the eggs by a factor of about 45, and as the original density was always about 10^8/ml., the density after dilution was $2–3\times10^6$/ml. At this sperm density, the incidence of polyspermy is negligible, being less than 2% (Rothschild & Swann, 1950).

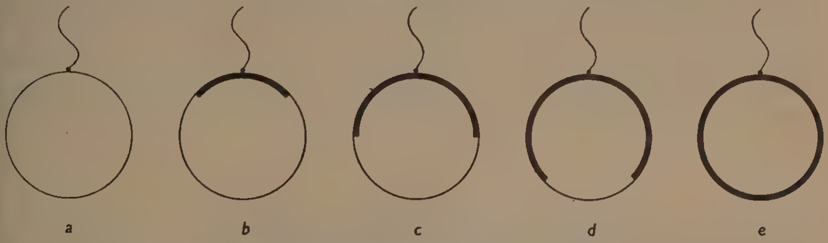


Fig. 1 *a–e*. Conduction of the block to polyspermy in an egg fertilized at $t=a$. The conduction time T equals $e-a$.

THEORY

The results and their interpretation are easier to follow if prefaced by a brief outline of the theoretical considerations upon which the experiments and analysis are based. Each experiment provides information showing how the proportion of polyspermic eggs in a suspension varies with the time of contact between eggs and spermatozoa. Five conditions of a fertilized egg are shown in Fig. 1 *a–e*. In *a* the egg has just been fertilized; *b* is a little later, the block to polyspermy having travelled part of the way round the upper half of the egg; in *c* half the egg is covered; in *d* three-quarters of the egg are covered; and in *e*, when $t=T$, the block to polyspermy has passed completely over the egg surface. It is clear from these diagrams that the chances of polyspermy in unit time are greatest immediately after the first fertilization, since from then on the area of egg surface available for re-fertilization gets smaller and smaller. Theoretically, there must therefore be a change in the form of the curve relating the proportion of polyspermic eggs to the time of contact with the spermatozoa, because at $t=T$, the conduction time of the block to polyspermy, there can be no further increase in the number of polyspermic eggs. The form of the theoretical curve is shown in Fig. 3. The abscissa at the 'point of intersection' between the curved and horizontal portions of this curve will be the conduction time of the block to polyspermy, T. To determine T and its precision, a curve must be fitted to the experimental points. The form of this curve cannot be determined from the experimental data; it must be based on some hypothesis. The hypothesis, which leads to equ. (1) on p. 480, is that when an egg is fertilized, a change in surface structure which makes the egg 'impermeable' to further spermatozoa passes over the egg at a uniform rate, unaffected parts of the egg surface remaining equally receptive to spermatozoa. We cannot prove that such a hypothesis is correct, but the experimental data may either show that it should be dismissed, or that there are insufficient grounds for rejecting it. The methods of fitting curves to the experimental points, of estimating the 'intersection point' T and its precision, and of testing the original hypothesis present problems of considerable difficulty. Details of the methods used will be found at the end of this paper in an appendix by Dr H. Ruben, to whom we are much indebted for devising a rigorous way of analysing the experimental data.

RESULTS

Fertilization at $t=0$. It was first necessary to establish that when eggs were mixed with high concentrations of spermatozoa, all the eggs were in fact fertilized in the neighbourhood of $t=0$. An experiment to verify that this does happen is shown in Fig. 2. This experiment was done by the 'hypo-hypertonic method' described in a previous paper (Rothschild & Swann, 1951*b*). The sperm density was somewhat lower than that used in the block to polyspermy experiments to be described; nevertheless, 50% of the eggs were fertilized in 1·25 sec., 94% in 2·5 sec. and 100% in 3·75 sec. It is therefore safe to assume that in all the block to polyspermy experiments, 100% fertilization occurred within 2 sec. of $t=0$, the time at which the eggs and spermatozoa were mixed together.

Conduction time of the block to polyspermy, T. Fig. 3*a* and *b* are graphs showing the variations in the proportions of polyspermic eggs after different sperm-egg interaction times ranging from 0 sec., when all the eggs were fertilized for the first time, to 180 sec. Each graph consists of the experimental points, the fitted curve based on equ. (1), p. 480, and the point on the curve at the ordinate at $t=T$, the conduction time of the block to polyspermy. These two experiments were deliberately selected to show a 'good' and an 'indifferent' result, from the point of view of correspondence between theory and experiment. The words 'good' and 'indifferent' will be quantitatively defined in the next section.

The results of all experiments are summarized in Table 1. The second column in this table gives the conduction times, T, of the block to polyspermy in all experiments; these are also shown as a histogram in Fig. 4. The third column gives the standard error of T. It will be observed that the block times, whose arithmetic mean is 63 sec., vary over a wide range from experiment to experiment. This is to be expected, as suspensions of eggs from different females often show marked variations in their properties (Rothschild, 1949*a*). One of us (R.) found two lots of eggs of *Echinus esculentus* which appeared to have no block to polyspermy at all, or an extremely slow one, in the same year as these experiments were done. The fourth column in Table 1 gives the values of α for each experiment. This figure is of great importance in the analysis of the results; it is a measure of the condition of the egg surface during the propagation of the block to polyspermy, and it can be directly compared with the α values obtained in the previous experiments on monospermic eggs (Rothschild & Swann, 1951*b*). The fifth column gives the standard error of α. The sixth column is identical with the fourth except that the α's have been divided by n, the sperm density relevant to the experiment in question. The values of χ^2 are tabulated in the seventh column, the number of degrees of freedom being given in brackets. Three of the fourteen experiments produced values of χ^2 which were sufficiently high to warrant rejection of the original hypothesis. These experiments are marked with an asterisk. One of them, Exp. 13, is also shown in Fig. 3*b*. All other experiments were 'good' in the sense that the results provided insufficient grounds for rejecting the original hypothesis.

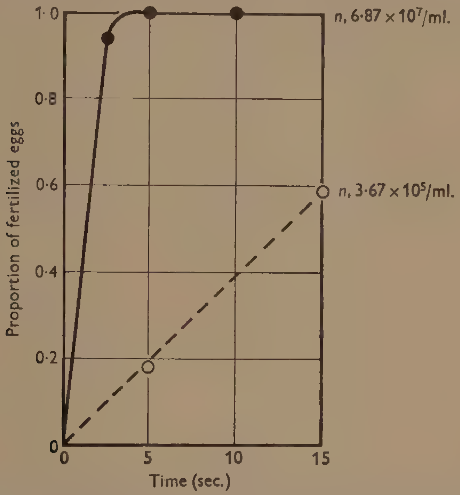
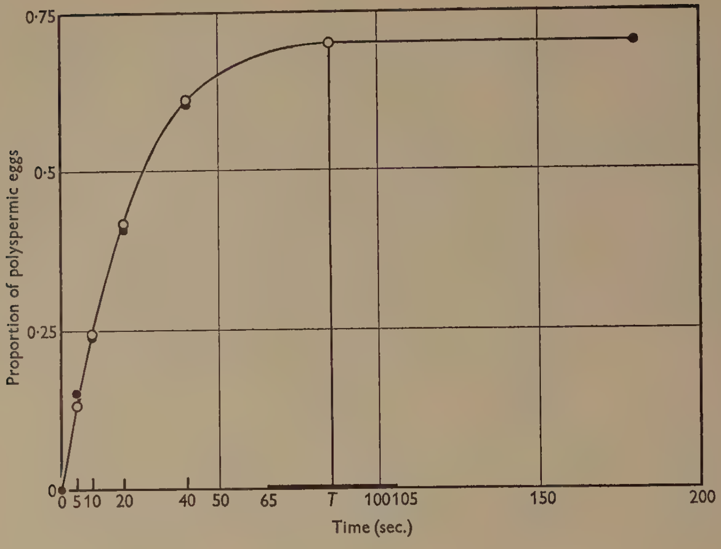
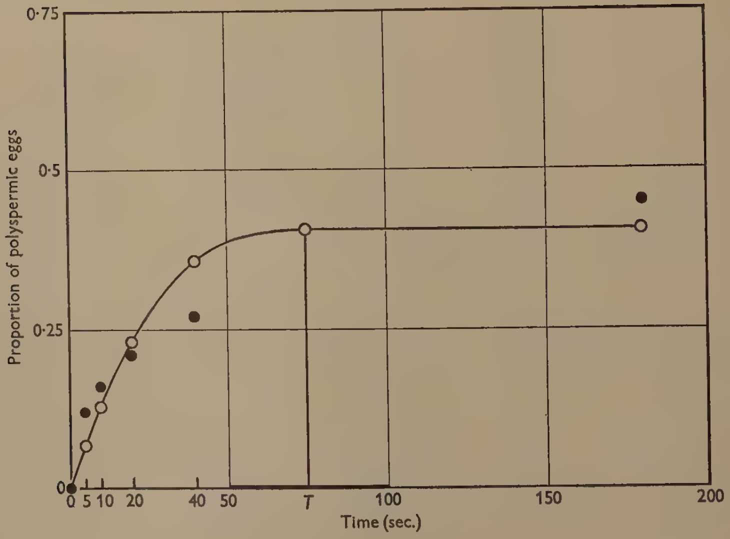


Fig. 2. Proportion of fertilized eggs in a suspension after various times of contact between unfertilized eggs and spermatozoa, at two different sperm densities.

Fertilization parameter α. The probability of a successful sperm-egg collision, p, is related to α by the equation $\alpha = Zp$, where Z is the number of spermatozoa colliding with the egg surface in unit time. The evaluation of Z requires an estimate of the mean speed $\bar{c}$ of the sperm suspension. Although this can be made with some confidence at low sperm densities, all the experiments described



a



b

Fig. 3. *a*. Proportion of polyspermic eggs in a suspension after various times of contact between eggs fertilized at $t=0$ and spermatozoa. Sperm density, $9{\cdot}11 \times 10^7$/ml.; ●, experimental points; ○, theoretical points. *T*, 85 sec. The thick line from 65 to 105 sec. on the time axis is the interval within which *T* lies (fiducial probability 0·90). *b*. The same as *a* but a 'bad' experiment (see text). Sperm density, $2{\cdot}80 \times 10^8$/ml. *T*, 75 sec.

Table 1

Exp. no.	T (sec.)	S.E. of T	α	S.E. of α	$\alpha/n \times 10^{11}$	χ^2
1	17	5	0·026	0·006	23·7	5·088 (4)
2	69	13	0·014	0·001	7·7	3·674 (4)
3	72	11	0·029	0·003	23·3	8·908 (3)*
4	74	11	0·030	0·003	27·1	3·152 (3)
5	94	14	0·025	0·002	30·0	4·199 (3)
6	85	12	0·028	0·003	30·9	0·580 (3)
7	26	5	0·035	0·002	25·7	2·212 (3)
8	60	10	0·026	0·008	22·3	3·494 (3)
9	31	6	0·034	0·005	24·5	3·673 (3)
10	91	15	0·022	0·002	12·0	5·415 (3)
11	85	14	0·033	0·003	17·1	6·206 (3)
12	34	6	0·085	0·012	43·1	12·827 (2)*
13	75	15	0·014	0·002	5·0	13·474 (3)*
14	67	14	0·015	0·002	4·9	6·220 (3)

T, conduction time of block to polyspermy; S.E., standard error; n, number of spermatozoa/ml.

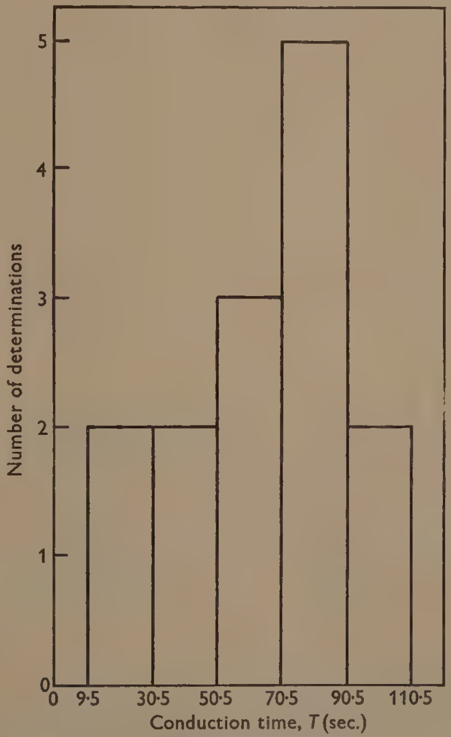


Fig. 4. The conduction times of the block to polyspermy as a histogram.

in this paper were carried out at high sperm densities, in which circumstances mean speeds cannot be accurately measured. For this reason, and because α is an 'observable' quantity, being the slope of the curve relating the logarithm of the proportion of fertilized eggs to the sperm-egg interaction time (first fertilization),* the results are given in terms of α rather than p in this paper.

In the previous paper (Rothschild & Swann, 1951*b*, p. 407), values of p, the probability of a successful sperm-egg collision, were tabulated for different sperm densities. These values have been converted into the corresponding α/n values† and plotted against $\log_{10} n$ in Fig. 5. Even if the first point, which had a large standard error, is ignored, there is a tendency for the points to flatten out at higher sperm densities. To compare these α/n values with those obtained in the experiments tabulated in Table 1, the α/n, n plot must be logarithmic, which may tend to reduce the curvature of the relationship between α/n and $\log_{10} n$ in Fig. 5. In such circumstances any discontinuity between α/n before and after the first fertilization might be difficult to see. The comparison is made in Fig. 6, and, in spite of the adverse effects of the logarithmic plot, it is clear that α/n during the propagation of the block to polyspermy *is markedly lower* than α/n before fertilization. This is not due to differences between the eggs in successive years, because α/n for the experiment in Fig. 2, which is shown as a triangle in Fig. 6, is precisely where it should be for the pre-fertilization α/n level.

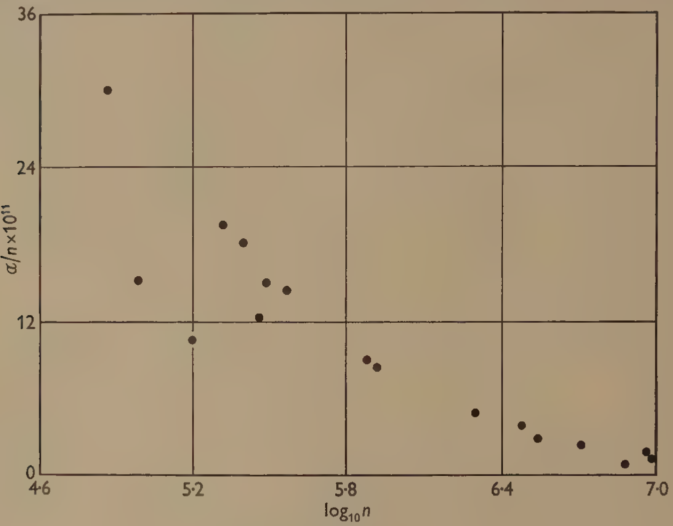


Fig. 5. For explanation see text.

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* The geometrical interpretation of α is less simple in this paper, though reference to equ. (2) on p. 480 shows that for t small, α is again the slope of the curve relating the logarithm of the proportion of polyspermic eggs to the sperm-egg interaction time.

† $\alpha/n = \pi a^2 \bar{c} p$, where a = egg radius.

The conclusions from these results are first, that there is a low-speed block to polyspermy, which takes some 63 sec. (arithmetic mean) to pass over the egg surface. Secondly, that the fall in α after fertilization and before the block to polyspermy is complete strongly suggests that there is a higher speed but partial block to polyspermy, which covers the egg surface in a shorter time than the final block which makes the egg completely impermeable to spermatozoa. Elsewhere, attention has been drawn to a feature of previous experiments which is suggestive in this connexion, the difficulty of reconciling a low-speed block to polyspermy with the small incidence of polyspermy observed at normal sperm densities. This question is discussed in greater detail in the next section.

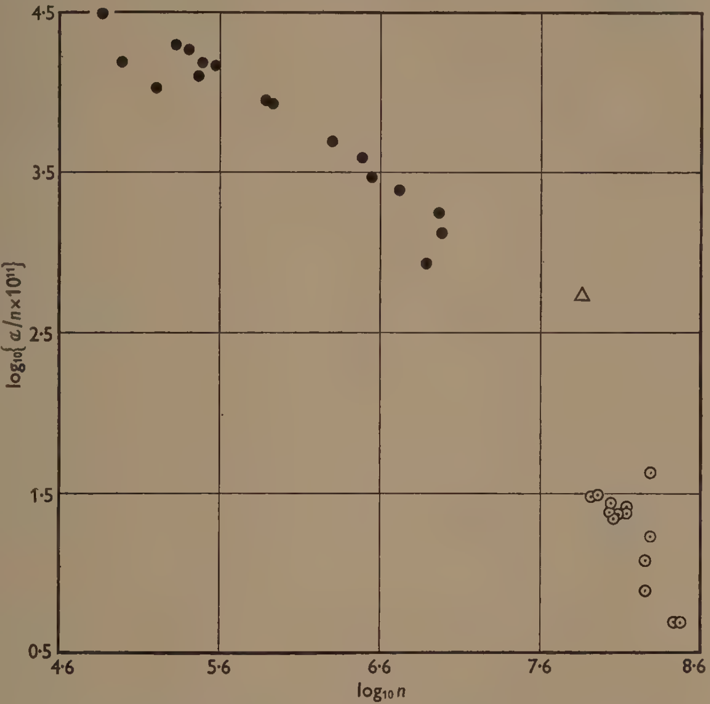

Fig. 6. For explanation see text. ● and △, first fertilization; ⊙, re-fertilization.

DISCUSSION

The original hypothesis, first mentioned on p. 472, involves several assumptions and simplifications which might make the fit between theory and experiment less good, or in other words make χ^2 rather high. One simplifying assumption is that the conduction rate of the block to polyspermy is uniform. The only change that has so far been observed, the cortical change, is not propagated over the egg surface at a uniform rate (Rothschild & Swann, 1949), and there is some evidence for believing that this non-uniformity is real (Rothschild, 1949 *b*). A further simplifying assumption is that all eggs in the suspension are fertilized exactly at $t=0$. Finally, we have assumed that *no* re-fertilization takes place after the dilution of the original sperm-egg mixture. Although these assumptions are individually justifiable and necessary as approximations, they may collectively produce deviations from theory. Any attempt to take these factors into consideration in devising an analytical procedure would make the already difficult calculations too complicated to warrant serious consideration.

The data tabulated in Table 1 make it reasonably certain that under the conditions of these experiments, the block to polyspermy takes between 17 and 94 sec. to pass over the egg surface, the variation being probably due to the varying properties of eggs from different females. This conclusion can also be reached, though not quantitatively, by an examination of the raw experimental data, as in Fig. 3*a* and *b*.

The position is not quite the same in regard to the high-speed block to polyspermy, the existence of which, as was said earlier, is strongly suggested by the fall in α during the conduction of the block to polyspermy. No high-speed block to polyspermy has been observed, from which it follows that its existence can only be the subject of surmise. The observations on the fall in α do however agree, in an interesting way, with our previous observations on the incompatibility of the observed incidence of polyspermy at normal sperm densities with a block to polyspermy which takes ten or more seconds to pass over the egg surface. Examination of Fig. 6 shows that if the pre-fertilization α/n values are extrapolated to $n=10^8$, the pre- and post-fertilization α/n values are in the ratio of 1:15. Bearing in mind that the experiments to determine α/n for unfertilized eggs were done on jelly-free eggs while those described in this paper were not (see Rothschild & Swann, 1951 *b*), the factor for the fall in α/n may be of the order of 20. This means that, taking a value of 60 sec. for the complete block to polyspermy, the proportion of polyspermic eggs in an experiment in which n equalled 10^8/ml. would have been reached in about $60/20=3$ sec., if α/n had not fallen by a factor of 20. This suggests that the first, incomplete, block to polyspermy must pass over the egg surface in less than 3 sec. If the conduction time of the first incomplete block to polyspermy were as much as 3 sec., the experimental 5 sec. points in the curves relating the proportions of polyspermic eggs to the sperm-egg interaction times should lie well above the theoretical 5 sec. points; while if this fast block were complete in a small fraction of a second, the experimental points should be evenly distributed about the theoretical curve. This is because α/n would be twenty times higher during the propagation of the fast block to polyspermy than after its completion. Table 2 is a comparison of the observed and theoretical 5 sec. values; there is a clear tendency for the experimental values to be higher than the theoretical ones. Because the experimental 5 sec. points are not *much* higher than the theoretical points, the conduction time of the fast but incomplete block to polyspermy must probably be less than 1 sec. In view of the simplifying assumptions referred to above, this argument about the conduction time of the incomplete block to polyspermy cannot at present be more than tentative.

The 'conditions of the experiment' were mentioned earlier on. The high sperm density used, which is essential in these experiments, may be responsible for two abnormal conditions. First, there may be an abnormally high concentration of the lytic substance A. III in contact with the eggs. This might make them unusually receptive to spermatozoa. In that case one would not expect α/n to be lower than in the pre-fertilization experiments, since, for a given sperm suspension, α is a direct measure of the 'receptiveness' of the eggs to spermatozoa. Furthermore, the experiment shown in Fig. 2, which occurs again as a triangular point in Fig. 6, is inconsistent with this explanation of the facts. Secondly, the high concentration of spermatozoa might cause a fall in the pH of the medium which in turn might produce an abnormal amount of polyspermy. The arguments against the A. III hypothesis probably apply in this case as well. Neither of these factors are easy to control in experiments of this type, though they can and should be the subject of further experiments. To sum up, these experiments suggest that the adhesion of the spermatozoon to the egg surface initiates a change which probably takes a second or somewhat less to pass over the egg surface. This does not make the egg completely impermeable to spermatozoa but reduces the probability of re-fertilization by a factor which may be of the order of 20. Simultaneously or immediately afterwards, what might be called a slow mopping-up process is initiated. The latter may well be intimately associated with the cortical change and even with the elevation of the fertilization membrane. The fact that eggs whose fertilization membranes have been removed cannot normally be re-fertilized does not necessarily conflict with this view, as by the time this can be attempted, far-reaching changes have started in the egg as a whole. In such circumstances successful re-fertilization would be improbable, though under certain special conditions re-fertilization can be achieved (Sugiyama, 1952).

Table 2. *Comparison of theoretical and experimental 5 sec. points in the curves relating proportions of polyspermic eggs and sperm-egg interaction times*

Exp. no.	1	2	3	4	5	6	7
Experimental proportion of polyspermic eggs	0·09	0·10	0·15	0·15	0·14	0·15	0·17
Theoretical proportion of polyspermic eggs	0·12	0·07	0·13	0·14	0·12	0·13	0·16
Δ	−0·03	+0·03	+0·02	+0·01	+0·02	+0·02	+0·01
Exp. no.	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Experimental proportion of polyspermic eggs	0·16	0·17	0·10	0·20	—	0·12	0·08
Theoretical proportion of polyspermic eggs	0·12	0·16	0·10	0·15	—	0·07	0·07
Δ	+0·04	+0·01	0	+0·05	—	+0·05	+0·01

The preceding arguments might be thought to imply that the rapid partial block and the slower complete block to polyspermy are distinct processes involving different mechanisms. They may, however, be different aspects of one continuous process—the rapid partial block representing a steeply falling part of the curve of α against time, and the slow complete block, a later and more gradual decline in the same curve. At present there is no evidence upon which to decide between these alternatives, though the earlier results on the effect of nicotine (Rothschild & Swann, 1950) require consideration in this context. Nicotine was found to increase the probability of a successful collision, during the period of the block to polyspermy, by a factor of about 20 at a sperm density of 10^8/ml. As nicotine did not increase sperm speeds or the conduction time of the cortical change, it must simply have raised α by a factor of about 20. The amount by which α normally falls, after the initiation of the block to polyspermy, has been shown in this paper to be 15–20. This raises the interesting possibility that there are two separate or separable blocks to polyspermy, the first but not the second of which is suppressed by nicotine.

APPENDIX

Estimation of α and T

BY H. RUBEN

From the Department of Genetics, University of Cambridge

The hypothesis on p. 472 may be shown to lead to the following relationship:

$$Q = e^{-g(t;\alpha,T)}, \tag{1}$$

where Q = proportion of monospermic eggs,
t = sperm-egg interaction time,
α = fertilization parameter (measuring the sperm-egg interaction rate),
and T = conduction time of the block to polyspermy.

The decay function g is defined by

$$\left.\begin{aligned} g(t;\alpha,T) &= \frac{\alpha T}{2\pi}\left(\sin\frac{\pi t}{T} + \frac{\pi t}{T}\right) \qquad (0 \leqslant t \leqslant T) \\ &= \frac{\alpha T}{2} \qquad\qquad\qquad\qquad (T < t \leqslant \infty). \end{aligned}\right\} \tag{2}$$

The combined likelihood of a set of independent samples is

$$L = \Pi \binom{r}{m} Q^m (1-Q)^p, \tag{3}$$

where m = number of monospermic eggs in any sample,
p = number of polyspermic eggs,
and $r = m + p$.

The maximum-likelihood estimates of α and T are obtained by setting the separate scores relating to the parameters equal to zero. The equations of estimation therefore are

$$\left.\begin{aligned} K_\alpha &= -\frac{\partial \log L}{\partial \alpha} = -\Sigma\left(\frac{m}{Q} - \frac{p}{1-Q}\right)\frac{\partial Q}{\partial \alpha} = 0, \\ K_T &= -\frac{\partial \log L}{\partial T} = -\Sigma\left(\frac{m}{Q} - \frac{p}{1-Q}\right)\frac{\partial Q}{\partial T} = 0. \end{aligned}\right\} \tag{4}$$

Using equs. (1) and (2), equs. (4) reduce to

$$\left.\begin{aligned} \frac{1}{2\pi}\Sigma\left(m-p\,\frac{Q}{1-Q}\right)\left(\sin\frac{\pi t}{T}+\frac{\pi t}{T}\right)&=0,\\ \frac{1}{2\pi}\Sigma\left(m-p\,\frac{Q}{1-Q}\right)\left(\sin\frac{\pi t}{T}-\frac{\pi t}{T}\cos\frac{\pi t}{T}\right)&=0,\end{aligned}\right\} \tag{5}$$

it being understood that the trigonometric factors in these equations are equated to π whenever $t>T$.

The appropriate information matrix $[I]$ is required for the evaluation of the sampling errors of the estimates $\hat{\alpha}$ and $\hat{T}$. For this purpose we need the expected values of the squares and product of the two scores. We have

$$\left.\begin{aligned} K_\alpha^2&=\Sigma\Sigma\left(\frac{m_i}{Q_i}-\frac{p_i}{1-Q_i}\right)\left(\frac{m_j}{Q_j}-\frac{p_j}{1-Q_j}\right)\frac{\partial Q_i}{\partial\alpha}\frac{\partial Q_j}{\partial\alpha},\\ K_\alpha K_T&=\Sigma\Sigma\left(\frac{m_i}{Q_i}-\frac{p_i}{1-Q_i}\right)\left(\frac{m_j}{Q_j}-\frac{p_j}{1-Q_j}\right)\frac{\partial Q_i}{\partial\alpha}\frac{\partial Q_j}{\partial T},\\ K_T^2&=\Sigma\Sigma\left(\frac{m_i}{Q_i}-\frac{p_i}{1-Q_i}\right)\left(\frac{m_j}{Q_j}-\frac{p_j}{1-Q_j}\right)\frac{\partial Q_i}{\partial T}\frac{\partial Q_j}{\partial T},\end{aligned}\right\} \tag{6}$$

the double summations to be taken over all combinations of pairs of samples. Since

$$\mathscr{E}\left\{\left(\frac{m_i}{Q_i}-\frac{p_i}{1-Q_i}\right)\left(\frac{m_j}{Q_j}-\frac{p_j}{1-Q_j}\right)\right\}=0\quad(j\neq i),$$

the expectations of the quantities in equ. (6) reduce, using equ. (1), to

$$\left.\begin{aligned} \mathscr{E}\,(K_\alpha^2)&=\Sigma r\left(\frac{\partial g}{\partial\alpha}\right)^2\frac{Q}{1-Q},\\ \mathscr{E}\,(K_\alpha K_T)&=\Sigma r\,\frac{\partial g}{\partial\alpha}\frac{\partial g}{\partial T}\frac{Q}{1-Q},\\ \mathscr{E}\,(K_T^2)&=\Sigma r\left(\frac{\partial g}{\partial T}\right)^2\frac{Q}{1-Q}.\end{aligned}\right\} \tag{7}$$

More explicitly, on using equ. (2),

$$\left.\begin{aligned} \mathscr{E}\,(K_\alpha^2)&=\frac{T^2}{4\pi^2}\Sigma r\left(\sin\frac{\pi t}{T}+\frac{\pi t}{T}\right)^2\frac{Q}{1-Q},\\ \mathscr{E}\,(K_\alpha K_T)&=\frac{\alpha T}{4\pi^2}\Sigma r\left(\sin\frac{\pi t}{T}+\frac{\pi t}{T}\right)\left(\sin\frac{\pi t}{T}-\frac{\pi t}{T}\cos\frac{\pi t}{T}\right)\frac{Q}{1-Q},\\ \mathscr{E}\,(K_T^2)&=\frac{\alpha^2}{4\pi^2}\Sigma r\left(\sin\frac{\pi t}{T}-\frac{\pi t}{T}\cos\frac{\pi t}{T}\right)^2\frac{Q}{1-Q},\end{aligned}\right\} \tag{8}$$

with the same interpretation of the trigonometric functions whenever $t>T$ as in equs. (5).

The elements in the information matrix are the quantities in equs. (8), i.e.

$$\left.\begin{aligned} I_{\alpha\alpha}&=\mathscr{E}\,(K_\alpha^2),\\ I_{\alpha T}&=\mathscr{E}\,(K_\alpha K_T)=I_{T\alpha},\\ I_{TT}&=\mathscr{E}(K_T^2).\end{aligned}\right\} \tag{9}$$

The estimates $\hat{\alpha}$ and $\hat{T}$ are jointly distributed asymptotically about their true values according to a bivariate normal distribution with a variance-covariance matrix which is the reciprocal of the information matrix. This provides a region (the interior of an ellipse) within which the 'parameter point' (α, T) may be said to lie for any level of fiducial probability. If $\chi^2_{2;1-\beta}$ is the critical value of χ^2 on 2 degrees of freedom at a probability level $1-\beta$, the estimation region is defined by

$$I_{\alpha\alpha}(\alpha-\hat{\alpha})^2+2I_{\alpha T}(\alpha-\hat{\alpha})(T-\hat{T})+I_{TT}(T-\hat{T})<\chi^2_{2;1-\beta}. \tag{10}$$

The sampling variances of $\hat{T}$ and $\hat{\alpha}$ in their marginal distributions are $I_{\alpha\alpha}/|I|$ and $I_{TT}/|I|$, and if we approximate by substituting the estimated values of α and T for their true values in the latter expressions, we obtain intervals within which α and T may be said to lie, corresponding to any level of fiducial probability.

Computation in equ. (5) is terminated when

$$\left|\frac{\Delta T}{S(T)}\right|\leqslant 0{\cdot}1, \tag{11}$$

where $S(T)$ is the estimated standard error of $\hat{T}$ and ΔT, the systematic error in $\hat{T}$, is given by

$$\Delta T=\frac{I_{\alpha\alpha}\Delta K_T-I_{\alpha T}\Delta K_\alpha}{|I|}, \tag{11·1}$$

ΔK_T and ΔK_α being the scores at termination. This ensures that the systematic error in $\hat{T}$ is sufficiently small compared with its sampling error.

To test the validity of the underlying hypothesis we define

$$\hat{Q}=e^{-g(t;\,\hat{\alpha},\,\hat{T})}. \tag{12}$$

On the assumption that the hypothesis is true, the quantity $\Sigma I(Q)(m/r-\hat{Q})^2$ is in large samples distributed approximately as χ^2 on $k-2$ degrees of freedom if k is the number of independent observations, two degrees of freedom having been lost in the estimation of α and T. $I(Q)$ is the estimated amount of information about the observed proportion of monospermic eggs, i.e.

$$I(Q)=\frac{r}{\hat{Q}(1-\hat{Q})}. \tag{13}$$

SUMMARY

1. The reactions of sea-urchin eggs (*Psammechinus miliaris*) to high concentrations of homologous spermatozoa have been investigated by a new method.

2. The method was to inseminate eggs with high concentrations of spermatozoa (10^8/ml.), which ensured that all eggs were fertilized for the first time at the beginning of the experiment; and then functionally to separate the eggs and spermatozoa at known times after the original insemination.

3. The proportion of polyspermic eggs in the suspension rises from zero at $t=0$ to a constant value at a time T, which is the conduction time of the block to polyspermy. In fourteen experiments on eggs from different sea-urchins, the estimated values of T were 17, 69, 72, 74, 94, 85, 26, 60, 31, 91, 85, 34, 75 and 67 sec. (arithmetic mean 63 sec.). The standard errors of the estimates of T, which are tabulated in the text, ranged from 5 to 15 sec.

4. These results confirm previous experiments which strongly suggested that the conduction time of the block to polyspermy was of the order of seconds; these

earlier experiments were incompatible with a block to polyspermy lasting a fraction of a second.

5. At the same time experiments in this paper made it possible to estimate the fertilization parameter or sperm-egg interaction rate α, during the conduction of the block to polyspermy, and to compare it with the pre-fertilization α. In a given sperm suspension, α is a measure of the receptivity of the egg surface to spermatozoa. During the conduction of the block to polyspermy α was found to be markedly lower (1/20) than in unfertilized eggs.

6. This suggests that at fertilization there is a fast but incomplete block to polyspermy, whose conduction time may be 1 sec. or less. This is followed by the slower block which finally makes the egg impermeable to spermatozoa. This also confirms previous observations on the small incidence of polyspermy at normal sperm densities, which is not consistent with the concept of a slow block to polyspermy.

One of us (R.) is indebted to the Medical Research Council for provision of a laboratory assistant.

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PRELIMINARY OBSERVATIONS ON THE MECHANISM OF CLEAVAGE IN THE AMPHIBIAN EGG

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(*Received* 5 *December* 1951)

(With Five Text-figures)

The formation of blastomeres by the newly fertilized egg has been studied by many authors, who have found the process a convenient avenue for attacking the general problem of cell division. By far the greater part of the work has been carried out on the eggs of marine invertebrates, and, in spite of their great suitability for most types of experimental embryological investigation, the eggs of Amphibia have not as yet been made much use of in this connexion. The nuclear phenomena attendant on fertilization and cleavage in the Amphibia have been rather fully described (see, for instance, Fankhauser & Moore, 1941), but a short note by Schechtman (1937) contains almost everything that we know about the cleavage of the cytoplasm, and the part played in it by cortical movements.

During the last two summers a few experiments have been made which show that the amphibian egg provides very favourable material for a direct attack on certain of the crucial problems of cell division, since not only is it rather easy to perform micro-surgical operations on the developing cleavage furrow in a manner which would be very difficult in the small eggs of most invertebrates, but the pigmentation of the surface allows the cortical movements to be followed precisely in time-lapse cinema films. These experimental opportunities have not yet been by any means fully exploited, but the preliminary experiments have already yielded certain results which it seems worth while to record.

The experiments were made with eggs of *Triturus alpestris* which were laid and fertilized normally in the aquarium. The eggs were observed in Holtfreter solution, either of normal strength, or diluted with an equal volume of distilled water.

OBSERVATIONS

The time of fertilization of the eggs being not exactly known, the onset of cleavage could not be foretold in advance. The first sign of it which is visible externally is the appearance of a thin dark line in the neighbourhood of the animal pole. This marks the beginning of the cleavage furrow and, as is made clear by cinema films, it is formed by a gentle movement of the cortex from each side towards the line. It constitutes the 'primary furrow' of Schechtman, and as he notes, it is soon replaced by a 'secondary furrow', in the formation of which the cortex condensed in the line begins to expand, so that the line broadens, becomes paler, and begins to sink into the egg.

A series of experiments was performed on eggs in which the primary furrow had just appeared or was in process of being transformed into the secondary furrow. After removal from the jelly capsule, an egg at this stage is contained in the vitelline membrane and has the shape of a sphere considerably flattened in the animal pole region, where there is a space between the surface of the egg and membrane. If the membrane is completely removed, the egg settles down as a rather flat bun-shaped object, far removed in shape from a sphere. Cleavage may nevertheless be performed quite well, the two daughter blastomeres tending to fall apart under their own weight. In most of the experiments to be described, the vitelline membrane was removed only from the animal half of the egg or in some cases was left more or less intact, being cut through only sufficiently to allow the operation to be performed; in such circumstances what remains of the membrane helps to hold the egg into a more nearly spherical shape.

(i) An opening was made into the egg in the region more or less in line with one pole of the spindle. This is near the equator of the egg in the usual embryological sense, which takes the animal pole as its main point of reference. (There is a possible ambiguity in terms to be guarded against here, since the axis of the spindle is perpendicular to the axis of the egg.) Through this opening some of the interior cytoplasm of the egg is extruded. In several of the experimental eggs this extrusion was assisted by raking out more cytoplasm with a needle until about a quarter of the internal contents of each half of the egg had been removed. After this had been done the egg was quite flaccid, appearing in every way as though its surface was too large for the material left inside it. The wounds made during the operation did not close and heal unless they were fairly small.

The cleavage continued apparently normally until the egg was cut into two blastomeres (Fig. 1). The same result occurred when openings were made over both poles of the spindle, so that both the first two blastomeres were partially emptied. Injured blastomeres usually died before the onset of the second cleavage would be expected.

(ii) In a number of eggs in which the furrow extended over only about a fifth of the diameter, two cuts were made through the cortex parallel with the furrow, in the positions shown in Fig. 2. As Holtfreter (1943) has described, the first result of a cut through the surface coat is a retraction of the latter; this is shortly afterwards followed by a contraction of the wound, which fairly rapidly becomes completely closed. A similar series of events was noted here. In some cases the cleavage continued more or less normally, at least as far as could be seen externally, but in some eggs the cleavage furrow appeared to be shallower and to extend less deeply into the interior in the region of the wound. In all cases the closure of the wound took place before the furrow had penetrated very deeply into the egg.

(iii) In a few eggs (only three) the first sign of the cleavage furrow was completely extirpated, the parallel cuts being located rather closer to it than those shown in Fig. 2. In two cases, in which the wound healed rather rapidly, the cleavage appears to be quite normal. In the third the wound healing was delayed, and it was noted that the cleavage furrow appeared on the lateral parts of the egg at a time when at the animal pole there was no sign of a furrow, the wound being still open.

(iv) In another small series of eggs cuts were made perpendicular to the furrow, as in Fig. 3. Some 10 min. later, while the cuts were still gaping open, the lateral parts of the furrow were visible outside the cut regions.

(v) In a number of eggs a needle was inserted and waggled from side to side under the region of the primary furrow, so as to cut any material connexion there might be (by way of astral fibres, for instance) between the cortex and the more deeply lying structures. Cleavage continued quite normally.

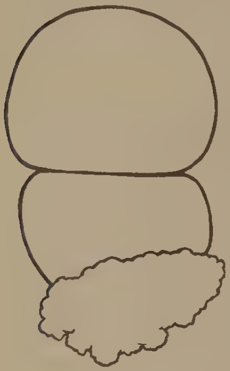

Fig. 1.

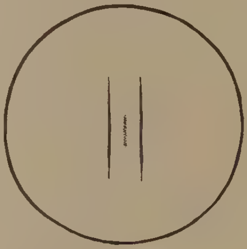

Fig. 2.

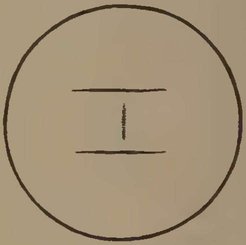

Fig. 3.

Fig. 1. Egg opened at equator (towards lower side of drawing) and some of the internal cytoplasm allowed to escape. Normal cleavage.

Fig. 2. Operation to an egg at the stage of the first sign of the cleavage furrow; cuts through the cortex parallel to the furrow.

Fig. 3. Cuts perpendicular to the furrow.

(vi) Two cuts were made, one on each side of the furrow, and located some distance away from it. By means of a needle, a path was cleared through the cytoplasm from one cut to the other, and a piece of cellophane carefully worked through so that it penetrated the egg from one side to the other. The thickness of the cellophane was approximately 0·08 mm., and the width of the strips used was about a quarter to a third of the egg diameter. The wounds through which the cellophane was passed naturally remained open, and cytoplasm extruded slowly through them. The cleavage furrow, however, continued to deepen, even though entirely separated from the interior by the strip of cellophane. Moreover, if the strip lay somewhat to one side of the furrow, the latter was observed to extend over it (see Fig. 4). So far as could be seen, the furrow extended downwards till it came in contact with the cellophane; unfortunately, it did not prove possible to fix and prepare the specimens for sectioning without so much injury that it was not possible to discover the conditions below the cellophane.

In the later development of these eggs, two phenomena were observed. After some time the cleavage furrow seemed to disappear over the region underlain by the cellophane. But this was somewhat obscured by the fact that the cellophane itself did not remain in place without disturbance. It was, in fact, gradually worked up towards the surface of the egg and eventually extruded completely. The mechanism of this action is obscure. It may be that the cut edges of the coat which lie underneath the cellophane tend to flow inwards below it, thus slowly pushing it out of the egg; it would appear probable, however, that some active reaction of the cell interior is also involved.

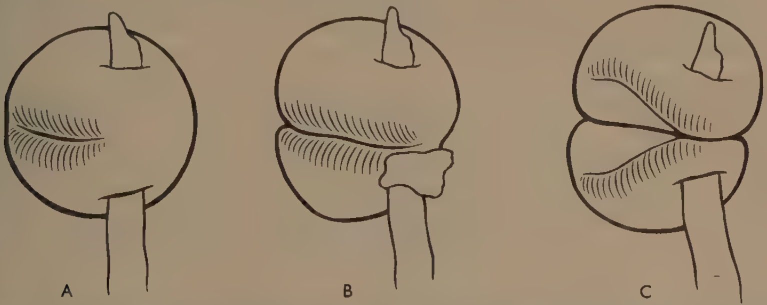

Fig. 4. A strip of cellophane inserted through an egg, not far beneath the cortex. A, at 3.23 p.m., a few minutes after the operation which was performed when the cleavage furrow was about half the length shown in the drawing; B, at 3.42 p.m.; C, at 3.50 p.m.

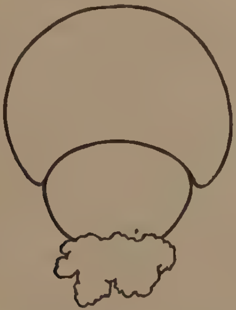

Fig. 5. Egg opened at equator (towards lower side of drawing) and an attempt made to pull the cleavage spindle towards the opening.

(vii) In another series of eggs a cut was made near the egg-equator, over what should be one of the poles of the spindle. With a needle, some cytoplasm was pulled out, and the needle moved in such a way as might be expected to rake the spindle towards the wound; since the egg is quite opaque this operation must be made 'in the dark', and there is no way of being certain that the desired result is achieved. In most cases, in fact, the cleavage continued with little change, but in one egg definitely, and in another less certainly, the extending cleavage furrow bent round so as to cut off a small blastomere containing the wound (Fig. 5).

DISCUSSION

The results even of these preliminary experiments would seem to show that certain theories of cell division which have been proposed in the past are not adequate for the amphibian egg. The experiments under § (i) demonstrate that internal turgor is not a major factor in this form, so that the astral gelation theory put forward some years ago by Gray (1924) for the echinoderm egg cannot apply here. The experiments in § (v) and particularly those in § (vi) make it equally clear that in this egg the cleavage can proceed in the absence of any material connexion between the cortex and the underlying spindle, so that theories which attribute an important role to the contraction of astral rays (such as that of Dan, 1948) must also be rejected.

It appears, in fact, that the formation of the furrow is a function of the cortex itself, since the first pigmented line of the primary furrow can deepen and become properly developed even in a region which has been isolated by means of a cellophane strip. The cellophane is, of course, permeable to small molecules, and it is not excluded that a diffusion of some substance from the spindle plays a part in the deepening of the furrow, but it appears rather doubtful whether diffusion would be rapid enough in the time available.

The capacity to form the furrow would seem to be fixed in the cortex some time before the furrow is actually visible. In the experiments in § (iv), for instance, the furrow appeared in lateral regions which were separated from the first primary furrow by transverse cuts.

The only evidence, in the present experiments, suggesting an influence by the spindle on the cleavage furrow is that of the few eggs described in § (vii) in which an attempt to move the spindle was followed by an alteration in the course of the furrow. General considerations derived from a study of the cleavage of eggs of many groups certainly suggest that the location of the spindle ultimately determines the position in which the furrow appears. These observations do not make it clear whether the influence of the spindle is exerted at the time the furrow is growing, or some time earlier. If further work confirms the present indication that the furrow deviates from its normal course if the spindle is moved during furrow-growth, one will have to conclude that the influence of the spindle is an immediate one. This can be reconciled with the evidence of the cellophane strip experiment either by supposing that the spindle's influence is mediated by an easily diffusible substance, or by supposing that it determines only the very early stage of the development of the furrow, which then has a certain autonomy in its growth both in depth and in length.

The few cinema films which have so far been taken with adequate definition to show the granules in the surface of the egg make it clear that rather extensive movements of the cortex occur both just before and during cell division. It seems probable that these play an important part in the mechanism of cell division, which can hardly be understood until they have been elucidated. Further work to this end is in progress.

SUMMARY

1. In experiments on eggs of the newt, *Triturus alpestris*, it was shown that the first cleavage is carried out normally when the internal turgor is completely relieved by the removal of a large fraction of the internal cytoplasm.

2. The furrow can extend and deepen when separated from the deeper parts of the interior of the cell by a strip of cellophane. Thus little or no part can be played by the contraction of astral fibres attached to the cortex. It seems probable that the factors immediately involved in the deepening and extension of the furrow are located in the cortex itself.

3. The bearing of these observations on some current theories of cell division is briefly discussed, and attention is drawn to the probable importance of general cortical movements, which have been detected but not yet analysed.

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ON THE EXISTENCE OF REGIONALLY SPECIFIC EVOCATORS

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(*Received* 24 *January* 1952)

Spemann (1931) pointed out that different parts of the organization centre in the amphibian gastrula have different properties, in that the more anterior regions tend, other things being equal, to induce the formation of more anterior parts of the neural system, such as the brain and associated sense organs, while the posterior parts tend to induce the neural tube of the trunk or tail. Living organizer grafts frequently become more or less fused with the induced tissues, so that if a definite organ is finally produced, the individuation of it involves both inducing and induced materials, the former playing the leading part. When dead pieces of the organizer are used as grafts, they cannot be similarly incorporated, and any individuation which may occur must have arisen entirely within the reacting material. It might still be possible, however, that the dead organizer was able to specify which region of the embryonic axis was represented by the induction, and thus guide the individuating processes. It has been known, however, since the work of Holtfreter (1934*a*) that organizer material after being killed loses its power to induce mesodermal tissues. It evocates only the appearance of neural tissue. If this becomes individuated into any recognizable organ, it is usually found to represent some region of the brain or eye, while the organs resulting from secondary inductive effects of the brain (e.g. nose or ear) are also frequently found. It is certainly not impossible to find a dead material which can induce more posterior regions of the axis, since Holtfreter (1934*b*), Chuang (1938) and Toivonen (1949, 1950) have demonstrated such activity on the part of dead adult tissues or extracts from them. No phenomena of the kind have, however, been described as resulting from the action of dead parts of the gastrula organizer, and it has been usual to consider that this material loses its trunk-inducing powers, and becomes solely a specific head-organizer, when killed.

The experiments to be described in this communication were carried out with the intention of discovering whether a very mild heat-treatment of organizer tissues would leave any posterior-inducing capacity still verifiable in them; and further to investigate the steps which led up to the formation of any specific organs which might be found. Experiments to this end have been carried out in a desultory way for some years, and more recently a fairly large series has been undertaken.

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* This work received financial support from the Agricultural Research Council, for which I wish to express my gratitude.

The material used was the newt, in most cases *Triturus alpestris*, although a few experiments have also been made with *T. palmatus*. The inducing material was taken from stages somewhat later than the gastrula, since it was thought that the regional differences would become stronger as development proceeds; it was taken from a number of stages between that of the open neural plate and that of the late tail bud. The donor embryos were immersed for a few seconds (not more than five) in water at about 90° C., and then transferred to cold water in which the anterior and posterior parts of the axis were removed to be used as inducers. These fragments of coagulated tissue were wrapped in flaps of ectoderm from young gastrulae with sickle-shaped blastopores, and the 'sandwiches' cultivated in Holtfreter solution in the usual way.

RESULTS

No sign of the induction of axial mesoderm was seen in any of the cultures.

Large masses of neural tissue were formed in nearly every case in which the implant was retained within the ectodermal flaps. It usually requires a good deal of imagination to assign such masses to any particular part of the normal neural axis. The eye is the organ which can, when it is well formed, be recognized with the greatest certainty. Eyes were produced with considerable frequency (about 30 %) by the explants containing anterior organizer regions, and an even higher frequency might perhaps have been found if the cultures, instead of being fixed at times suitable to exhibit the stages of individuation, had been cultured for longer till histological differentiation was more advanced. From the explants containing posterior pieces of the organizer, eyes were not produced at all in these experiments. Although in the literature there are many cases of the induction of eyes by implants of dead posterior organizers into whole embryos (e.g. Holtfreter, 1934*a*, table 10), or pieces of dead anterior organizer isolated in ectodermal sandwiches (e.g. Okada & Hama, 1944; Lopaschov, 1935, 1936), there appears to be no record of the induction of an eye by a dead posterior organizer in isolated ectoderm. It may be that this really does not occur, but we shall see later that it may not be the direct indication of a specific difference between anterior and posterior dead organizers which it seems to be at first sight.

It seems better merely to describe the types of inductions which occur at stages of differentiation corresponding to the late tail bud or early swimming larva, rather than to attempt to assign them to particular parts of the nervous system.

Type 1. These are large neural vesicles, with a rather extensive lumen, and walls varying from thin to moderately thick. They are the most brain-like in appearance, and indeed in many cases there is no doubt that they do actually represent some part of the brain. An epiphysis may sometimes be found attached to them. They may be accompanied by eyes, such as those described above, and in other cases there may be placodes or other neuralized thickenings still attached to the overlying epidermis in their neighbourhood. Some of these placodes undoubtedly represent nasal pits. There are other examples, however, in which such secondary structures are absent, and it is only the morphology of the neural vesicle itself which reminds one of the brain (Pl. 20, figs. 3, 4).*

Type 2. The neural structures may take the form of a mass of contorted, thin-walled tubules (Pl. 20, fig. 5).

Type 3. Masses of well-developed neural tissue, with a narrow lumen, and walls ranging from moderate to excessive in thickness (Pl. 20, fig. 6).

Type 4. Thick solid masses of neural tissue with no lumen (Pl. 21, fig. 1).*

Type 5. Whole mass neuralized, there being no epidermal covering (Pl. 21, fig. 2).

Type 6. Small neural tubes embedded in a mass of neuralized ectoderm (Pl. 21, fig. 3).

Type 7. Similar small tubes, but lying in a projection with a well-formed epidermis covering them (Pl. 21, fig. 4). Such tubes often pass into a mass of neural tissue which retains its continuity with the superficial epidermis, and the base of the projection containing them may also be filled with a similar neuralized tissue (Pl. 21, fig. 5).

Type 8. Masses of neuralized cells in which no well-defined tubules are formed. They may be clothed in a definite epidermis, or pass gradually into a solid mass of non-neuralized ectoderm.

Before attempting any further evaluation of these inductions, it is as well to examine the appearances presented by similar experiments at earlier stages. In general, the earlier the stage, the more chaotic the induced tissues are likely to be. We frequently find the whole interior of the explant full of a complex of neural vesicles and tubes, with larger plate-like areas lying against the more extended surfaces of the dead implant (Pl. 20, figs. 1 and 2). At very early stages one can sometimes find a symmetrically folded neural groove, but in the immediately succeeding stages it is rare to find any formation which bears much resemblance to the normal neural system of similar age.

DISCUSSION

The chaotic arrangement of the induced neural tissue in its early stages, and the great variability of its appearance later, suggests that in these inductions by dead parts of the organizer we are not in fact dealing with any sort of specific regional influences at all. It seems more likely that the implant merely evocates masses of neural tissue, but that if some region of the mass happens to have a shape roughly like that of some part of the neural system, forces arise within it which cause it gradually to approach that part in its development. This would amount to the spontaneous self-individuation of particular organs, arising on the basis of chance approximations to some normal arrangement.

There is a good deal of indirect evidence which makes it plausible to suggest that such self-individuation can occur. It would, of course, be a phenomenon related to the usual developmental tendency to 'regulate' defects, and thus not unexpected on general grounds. It is probably significant also that on looking through a series of sections of such inductions, one can frequently find places where the appearances strongly resemble the cross-section characteristic of some part of the neural tube; but the neighbouring sections usually show that the shape is not normally extended longitudinally, and that one is dealing with a vesicle rather than a tube. The fact that the characteristic cross-sectional patterns are produced at all under these circumstances indicates that they are in some way equilibrium forms, and suggests that complete organ shapes may similarly be shapes to which any not too abnormally formed neural mass of appropriate size will tend.

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* For Pls. 20 and 21 see following article.

Essentially this conclusion was reached by Barth & Graff (1943), who studied the effects of freeze-dried anterior and posterior parts of the axis of the neurula when grafted into explants of isolated ectoderm. They also found that no mesodermal induction took place, and that the neural tubes or neural masses induced had no definite structure. They did not cultivate their explants very long, and perhaps for that reason do not seem to have seen any specific organs such as eyes.

If such an interpretation is adopted, it is still necessary to explain why the self-individuation to brain regions occurs more often than to shapes characteristic of the trunk, and why these induced brains are produced more often by anterior than by posterior implants. With regard to the first question, there would seem to be two factors to take into account. One is the shape of the inducing implant, which presumably controls the original shape of the evocated neural rudiment. Most of the implants were either roughly square, or had a cubical or thick-cylindrical form. They had no part which would seem likely to induce a long narrow band of neural tissue, from which an elongated neural tube might have been expected to arise. Secondly, there seems no doubt that the characteristic shape of the trunk neural tube is causally connected in some way with the presence of mesoderm; those inducers which produce posterior inductions (e.g. in the experiments of Chuang and Toivonen) appear always to call forth mesoderm as well as neural tissue. It seems likely that the mesoderm is essentially involved in the elongation of the neural system, and that this in its turn is decisive for the assumption of the characteristic neural tube shape. Yamada (1950) has been led by such considerations to suggest that posterior induction always involves a structural factor in the inducer, and although it seems difficult to make his hypothesis in its original form cover the induction of tails by coagulated embryo extract (Holtfreter, 1934*b*), there seems no doubt that dynamic factors are more important for the morphogenesis of the posterior than for that of the anterior part of the body. In the absence of either an elongated inducing substratum or of the appropriate dynamic tendencies, the formation of neural vesicles with a cross-section similar to that of the neural tube may be as close an approximation to the trunk neural system as we have any right to expect. There is no clear evidence that such neural vesicles are induced more often by posterior than by anterior implants.

The reason for the greater frequency of brain inductions by anterior implants is probably to be found also in the influence of the shape of the implant on that of the induced neural tissue. The anterior implants frequently consisted of one half of the forebrain, and thus had a smooth surface of comparatively large area, which one might expect to induce a single broad neural plate which would start with a certain resemblance to the brain region at a similar stage. The more equidimensional shape of the posterior implants (except those taken from open neural plate stages) would be more likely to induce a number of separate smaller rudiments.

The tendency for more definite organs to be induced by implants grafted into whole embryos than by those enclosed in isolated flaps of ectoderm is probably also connected with the fact that in the former the area of contact between the inducer and the reacting tissue is more regular than it can be when the ectoderm is free to contract into random folds, as it does in explants.

The evidence described in this paper does not, of course, suffice to disprove the hypothesis that there may exist evocators which specifically induce some definite part of the embryonic axis. All that has been shown is that in the fairly early larval stages such specific influences are not strongly developed, or are destroyed by slightly elevated temperatures, and that the facts can be adequately explained without postulating them. The most apparently convincing evidence for the existence of such regionally specific substances (or 'modulators' as I have (1937) proposed calling them) is that derived by Chuang and Toivonen from implantations of dead adult tissues of various kinds. Both these authors have shown how the regional properties of such implants change after the application of various treatments, and Toivonen (1950) and Kuusi (1951) have carried the chemical study of them some distance. It is notable, however, that evocators which produce posterior structures always also produce considerable amounts of mesoderm. In fact, as far as one can judge from the published descriptions, the posterior quality is strictly proportional to the quantity of mesoderm; with much mesoderm the induced axis becomes a tail or trunk, with less it forms the hindbrain accompanied by ears, with none the archencephalic region of the brain. It seems quite possible, therefore, that the primary action of all these tissues and extracts is to evoke merely a mass of tissue with a certain proportion of mesoderm and neural plate, and that the individuation of this mass, so that it develops into a definite organ, is always a self-individuation, taking place within the evoked material in a manner determined by the mesodermal-to-neural ratio. If this were so, it would strictly be incorrect to speak of regionally specific evocators at all. We should be dealing only with mixtures of neural and mesodermal evocation. Only the future can tell, however, whether this suggestion, which now appears to be the simplest admissible, will prove to be correct.

SUMMARY

1. Pieces of the embryonic axis, taken from the anterior and posterior regions of embryos from the open neural plate to the late tail-bud stages which had been coagulated by a few seconds' immersion in water at 90° C., were inserted into flaps of gastrula ectoderm which were then cultivated in Holtfreter solution. No

induction of mesoderm occurred, but neural tissue was evoked in a high percentage of cases.

2. In early stages the neural tissue usually formed a more or less chaotic tangle of tubes and rods. At later stages it assumed a variety of forms, some of which were similar to parts of the brain, and such brain-parts might be accompanied by secondary structures such as eyes, nasal pits, ears, etc. No elongated tubes resembling the trunk neural tube were seen, although certain neural vesicles may have a cross-section very like that of the neural tube.

3. The induction of recognizable brain or eye was not uncommon when anterior implants were used, but was not seen at all with posterior implants. There was no other difference between the two sets of experiments.

4. It is suggested that the appearance of such organs is not due to the direct action of a regionally specific inducing factor, but rather that all such definite forms arise by a process of self-individuation which occurs within the induced mass of neural tissue. The direction this self-individuation takes, and thus the nature of the organ finally formed, is supposed to depend on chance resemblances between the mass and shape of parts of the original chaotic mass and some part of the normal embryo. It is argued that this could account for the apparently specific effect of the anterior implants.

5. In other experiments in which mesodermal tissues are also induced (e.g. with implants of adult tissues) it is likely that these take part in the self-individuation processes and tend to direct these towards the formation of posterior organs such as trunk and tail.

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THE EFFECT OF TYPE OF CONTACT WITH THE ORGANIZER ON THE NATURE OF THE RESULTING INDUCTION

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(*Received* 24 *January* 1952)

(With Plates 20 and 21 and Five Text-figures)

The result of the activity of the primary organization centre in the amphibian gastrula is to determine a complex organized pattern of development. Within the ectoderm there arises a neural system in which, at the early stages, one can distinguish at least three different developmental types—brain, neural tube and neural crest; another part of the animal hemisphere becomes mesoderm, and this can also be the result of an inductive action, since transplanted presumptive ectoderm can be caused to develop in this manner. There is general agreement that one cannot attribute these different end-results to any great extent to differences in the competence of the ectoderm submitted to the induction. There therefore remain two other factors to be investigated: differences either of a quantitative or qualitative nature within the organization centre, and differences in the type of contact between the various regions of the animal hemisphere and the organizer by which it becomes underlain. Waddington & Yao (1950) pointed out that there must be differences within the mesoderm in the rate at which evocating power is developed and in its final intensity, and they drew particular attention to the possible importance of the varying lengths of time during which the more anterior and the more posterior parts of the future neural system are in contact with inducing material.

The existence of a medio-lateral gradient of inducing power within the mesoderm was demonstrated by Waddington (1936), and more recently by Raven & Kloos (1945), while the reality of an antero-posterior gradient has been supported particularly by Dalcq (cf. Dalcq & Pasteels, 1937; Dalcq, 1947). This paper is concerned not so much with these gradients, but rather with the second of the factors mentioned above, namely, the duration and mode of contact between ectoderm and inducing substratum.

The subject has previously been rather little investigated. Under the inspiration of the gradient theory of Dalcq & Pasteels, Damas (1947) and Gallera (1947) have studied the effects of a precocious interruption of the contact between the ectoderm and its substratum, the latter confining his attention to the prechordal part of the

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* This work received financial support from the Agricultural Research Council, for which I wish to express my gratitude.

† Agricultural Research Council Scientific Staff.

neural plate and the former dealing with the remainder. Both authors found clear evidence that the process of induction is progressive and not of an 'all-or-none' nature, and that with a reduction in the time of action of the evocator there is a tendency for the presumptive neural plate to develop into derivatives of the neural crest, which must therefore be taken as representing a weaker response. The experiments of the two authors were, however, not very extensive, and did not give any conclusive evidence either of the existence of a medio-lateral gradient within the mesoderm or of any effect of the period of contact on the level of the antero-posterior axis of the resulting induction.

The first two groups of experiments to be described here (§§ 1 and 2) were in some ways the reverse of those of Damas and Gallera, in that the time of contact between the ectoderm and archenteron roof was prolonged beyond the normal. If this duration plays an important part in determining the different results of induction along the antero-posterior axis, one would expect that the longer the contact, the more posterior the level of the resulting induction, presumptive brain becoming converted into actual trunk neural tube; while the strongest action of this kind might be the conversion of presumptive neural plate into trunk mesoderm, which in normal development undergoes the longest period of being underlain by an inducing substratum. The nature of the stimulus which brings about the induction of mesoderm is a matter of great importance, because the fact that the primary organizer can induce two such different tissues as axial mesoderm and neural tissue is one of the most convincing arguments against the view that its activity is confined merely to the provisions of a unspecific stimulus. The experiments in §§ 3 and 4 therefore record a few other observations on the nature of the type of contact with the organizer which is necessary to convert presumptive ectoderm into mesoderm.

MATERIAL AND METHODS

Embryos of *Triturus alpestris* were used in nearly all the experiments. The only exceptions were the use of *T. vulgaris* in a small number of control explants in § 1, and in one explantation experiment (OY 25), and of *T. palmatus* in a few in § 3.

Operations were carried out under sterile conditions, using Holtfreter saline with sodium sulphadiazine added as a bactericidal agent, and the pH adjusted to 7·0 with a phosphate buffer solution.

Whole embryos were cultured for 5 days, up to the gill-bud stage (Harrison's stage 23–24). Explants were cultured for from 5 to 9 days, until they showed differentiation externally.

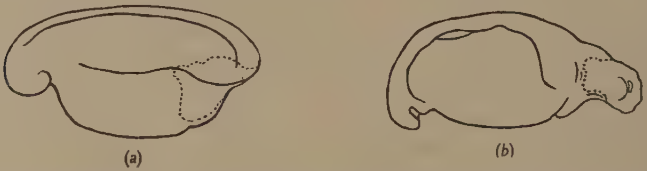
Most specimens were fixed in Bouin's fluid. Those which had been vitally stained with Nile Blue were fixed in Zenker's-acetic, left for 2 hr. in 2 % phosphotungstic acid, then dehydrated in alcohol containing 0·1 % phosphotungstic acid. This preserved the blue colour in whole specimens, but it was not afterwards visible in sections.

EXPERIMENTS

§ 1. *Effect of prolonging the time for which presumptive anterior neural plate has anterior archenteron roof passing underneath it*

In the first group of experiments a piece of presumptive anterior neural plate was transplanted from a late gastrula where it had already been underlain by anterior archenteron roof, to the anterior neural plate region of a younger gastrula. The graft was placed just anterior to the advancing archenteron roof of the host, so that it would immediately have anterior mesoderm passing beneath it for a second time.

The donor was usually at the small yolk-plug or slit blastopore stage (H 12–12$\frac{1}{2}$), but in a few cases a very early neural plate stage (H 13) was used. The host was a mid-gastrula (H 11–11$\frac{1}{2}$), with a horseshoe-shaped or large circular blastopore, except for ten cases where an early gastrula (H 10) was used.



Text-fig. 1. Differentiation of ectoderm graft (*a*) at neurula, and (*b*) at tail-bud stage (see text). Area occupied by graft indicated by dotted outline.

In the forty-eight experiments of group (*a*), the graft was left to develop *in situ*. In group (*b*) (thirty-eight cases), however, the graft (which had previously been stained in Nile Blue for identification) was removed as soon as the host had completed gastrulation. It was explanted with the archenteron roof underlying it, so that there should be no cutting short of the time for which induction went on. A piece of ventral ectoderm from the host was also included in the explant, to ensure that the mesoderm was completely enclosed. Under these conditions the tissue had a chance to develop independently, without being forced to go through the same neurulation movements as the host. In group (*c*) young gastrula ectoderm was placed for some time in contact with the archenteron roof of a mid- to late-gastrula, and then transplanted back to a young gastrula so that it then underwent the whole normal period of inductive action.

(*a*) *Differentiation of neural plate grafts left* in situ *on the host.*

In a few of these experiments the graft was stained in Nile Blue in order to ascertain that it had reached the desired position in the host at the end of gastrulation. In six out of seven cases investigated, the graft formed the anterior neural folds of the host (e.g. Text-fig. 1 *a*). At a later stage, it was seen to be forming part of the brain and the head epidermis (Text-fig. 1 *b*). In the seventh case, the graft had come to lie in the ventral head region, and had formed epidermis. This particular graft originated from a mid-gastrula, and was placed in an early gastrula host.

There were nineteen cases where the fate of the graft was clear without vital staining. One of these, which had been taken from a slightly older donor (early neural plate stage), had already formed a neural fold when the host was still a late gastrula: it later differentiated into a secondary brain lateral to the host's. Clearly both the tempo and the 'regionality' of its development were determined by its previous position in the donor.

There were two other embryos in which the graft had formed a secondary brain in a dorsal position, but its differentiation was synchronous with the host's—i.e. occurred later than if it had been left in position on the donor. The donor in these two cases had been a late gastrula.

(Ten grafts had formed a secondary brain in a ventral position. In one of these the secondary brain had the opposite orientation to the host's: this was from a donor at the early neural plate stage. In all the others the secondary brain had taken on the same orientation as the host's. In these ten instances it was probable that the graft had not been underlain by host archenteron roof, owing to having been placed too far anterior: these cases served, however, as controls to demonstrate the normal self-differentiating powers of the transplanted tissue.)

In six embryos the final position of the graft was indicated by an oedematous swelling: antero-ventral in four, and lateral in the other two. One of these on sectioning showed a slight thickening of the epidermis, which may have represented the beginnings of neuralization.

In twenty-four embryos the graft could not be identified at the gill-bud stage. In two of these doubling of the host brain had occurred, and in another two the posterior axis was bifid, but these abnormalities resulted from wounds in the mid-dorsal line. In another eight embryos the brain appeared enlarged, but there was no sign of extra graft tissue in sections. The remaining twelve embryos appeared perfectly normal, so that it could only be concluded that the graft had been incorporated into the host's neural plate. In these twelve cases the graft had been taken from a late gastrula.

If we eliminate all cases in which the graft was eventually found in a ventral position and had perhaps been placed too far anterior, there remain thirty-seven in which the graft developed into brain, and one in which it became epidermis. The ages of the donors and hosts of the former are given in Table 1. The single

Table 1. *Transplantation of presumptive neural plate from old donors to young hosts*

(All grafts developed into brain with the exception of one which gave epidermis.)

Donor stage	Host stage		
	Early gastrula	Horseshoe blastopore	Large yolk plug
Large yolk plug	—	6	—
Small yolk plug	4	4	6 (1 epidermis)
Slit blastopore	—	4	—
Early neural plate	3	10	—

graft yielding epidermis was from a small yolk-plug stage into a large yolk-plug stage.

There was certainly no evidence in any of these experiments that the degree of prolongation of the action of the organizer which was achieved could convert the presumptive neural plate into mesoderm, and there was very little suggestion (which will be discussed later) that it had even any effect on the 'regionality' of the grafted tissue.

(*b*) *Differentiation of neural plate grafts after explantation*

To gain an idea of what proportion of neural plate explants might be expected to develop successfully, twenty-eight control explants were made of pieces of presumptive anterior neural plate and underlying archenteron roof from a late gastrula-early neurula (H 12½–13), with ventral ectoderm to facilitate healing. Fourteen of these formed a definite brain, one formed neuroid tissue only, and eleven did not differentiate into any nervous tissue. The latter rather unexpected result may have been due to the small size of the explants.

Table 2

Donor	Explant's host	Total explants	Brain	Neuroid	Neural tube	No differentiation
Slit blastopore	Horseshoe blastopore	33	15	2	1	16
Slit blastopore	Large yolk plug	5	2	—	—	3

The explants of tissue which had previously been grafted into a gastrulating host were of necessity a little smaller than the controls, since part of the border of the stained graft was usually cut away when making sure that no host (unstained) neural plate was included. The underlying archenteron roof and a piece of ventral ectoderm from the host were included, as in the control explants. The results are summarized in Table 2.

These explants differentiated into a definite brain, with sense organs, in a high proportion of cases (44·7 %). Most of the brains were clearly recognizable externally after 7–9 days' culture. Any in which it was uncertain whether brain had formed were sectioned. Plate 21, fig. 6, shows one in which brain tissue was identified after sectioning. Of the explants 50 % had failed to differentiate and two had formed only neuroid tissue.

It was clear from external observations that it was always the blue-stained graft tissue which formed the brain in each explant: the host ectoderm remained as epidermis. In one case the host epidermis had completely enclosed the graft tissue, but even so, it had not been induced to form brain: the graft tissue, on the other hand, developed as brain within the explant.

It was quite clear from these experiments that the graft never developed otherwise than according to its presumptive fate, determined by its position on the host at the end of gastrulation. None of the donors used was more advanced than a late gastrula, and the graft had apparently always undergone 'neighbourwise' differentiation. For instance, in two cases where the graft was misplaced and finally came to lie in the middle of the host neural plate instead of anterior, a neural tube as well as a brain had developed. The archenteron roof has formed a notochord in one case, somites in the other. These were the only two explants which showed any differentiation in the mesoderm; in all the others it formed a solid mass in the centre. This mass represented only host mesoderm; there was no evidence that the graft has developed abnormally into mesoderm. On the contrary, it could always be seen to remain on the surface of the explant, either differentiating into brain or forming half the epidermal covering.

(*c*) *Submission of young ectoderm to a mid-gastrula organizer before normal gastrulation*

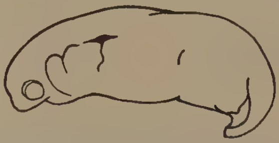
It seems likely that inducing activity of the archenteron roof does not rise instantaneously to its full intensity immediately after invagination, and it is therefore probable that rather little effect will be exerted on the ectoderm during the first stages of gastrulation. In the experiments just described, the excess of organizer action occurred after the normal process of gastrulation, and this might be comparatively ineffective. In another small series of eight experiments, a piece of presumptive ectoderm from a young gastrula was grafted over the anterior archenteron roof of a mid-gastrula with a horeshoe-shaped blastopore, and allowed to remain there until the small yolk-plug stage, when it was removed (with no attached mesoderm) and grafted a second time into the presumptive anterior neural plate of another young gastrula, where it was left to differentiate. Its normal period of submission to the action of the organizer was therefore preceded by an interval in which it had been acted on by the archenteron roof of fairly late gastrula stages.

Again, in none of these cases was there any sign that the graft had been induced to form mesoderm. In life, the grafts, which had been stained with Nile Blue, could be seen to form neural tubes; they began their infolding precociously, at a time when the host tissues were still an open plate. In the sections of late tail-end embryos, they could, in fact, be recognized as forming neural structures in the brain region of the host, except for one in which the graft lay in the anterior trunk of the host and became neural tube rather than brain. Even in those grafts which lay near the host's brain, the graft tissues had a more 'posterior' histological appearance than the host tissues against which they lay (Pl. 21, figs. 7 and 8). Thus the cells, although older than those of the host, contained more yolk granules, and the nuclei were less elongated, while the development of fibre tracts was less advanced. All these are characters both of younger neural tissue, and of more posterior as opposed to more anterior tissue. It is perhaps possible that the injury consequent on the operation might cause some delay in the differentiation of the graft, but there is no reason to expect that it would cancel and indeed overcome the difference in age. It seems more probable that one is confronted with a real tendency of the tissue submitted to prolonged organizer action to develop towards neural tube rather than brain, at least histologically. Such a tendency was less clear in the general morphology of the grafts. During life, it was seen in several cases that the graft began to roll into a neural groove and tube in advance of the surrounding host neural plate. This might indicate a more posterior character, since the neural tube closes earlier in the anterior trunk region than in the brain; but it might equally be merely an expression of the difference in age. In the embryos, as finally fixed in the late tail-bud or early swimmer stages, the grafts were in some cases separate from the host neural system, and then formed vesicles whose morphology was not very characteristically either that of the brain or of the neural tube. In other cases the graft was to some extent incorporated into the host brain, but was never morphologically quite normal, being thicker and more lumpy. It did not, however, develop into a clear-cut elongated neural tube.

§ 2. *Regional differentiation of the nervous system independently of the time for which parts of it have been underlain by organizer tissue*

(*a*) *Substitution of presumptive ectoderm from an early gastrula for presumptive neural plate of the late gastrula*

The whole of the presumptive neural plate was removed from thirty late gastrulae, and presumptive ectoderm (stained with Nile Blue in twenty-four cases) from the early gastrulae put in its place. It was then observed whether the grafted tissue would form a normal neural plate; this would only occur if the process of individuation was able to operate after the end of gastrulation. The conditions of the experiment prevented there being any difference in the length of time for which different parts of the ectoderm had been underlain by mesoderm, and also avoided the possible influence of any property peculiar to the mesoderm while it was still invaginating.



Text-fig. 2. Normal embryo formed after substitution of young gastrula ectoderm for presumptive neural plate at late-gastrula stage.

Twelve of the operated embryos developed into normal tail-bud stages (Text-fig. 2). In seven of these the graft had been stained, and it was seen to have formed the whole neural axis in five cases, though there were two cases in which it occupied only the brain region.

The most frequent abnormality among the other embryos was an incomplete healing of the grafted ectoderm, so that all types of distorted and doubled embryonic axes resulted. In nine of these, however, the graft had formed an axis which was at least differentiated into recognizable head and tail portions, though these were either reduced, or duplicated in other places by host tissue.

Table 3 gives a summary of these results. There was no question but that the presumptive ectoderm was able to develop into a regionally differentiated neural axis, even though it had never experienced archenteron roof actually invaginating beneath it.

(*b*) *Antero-posterior reversal of the presumptive neural plate at the late gastrula stage*

In nineteen late gastrulae (H 12–12½) the presumptive neural plate ectoderm was reversed so that its originally anterior part, which had been underlain by archenteron roof for less time than other regions, now lay posteriorly, while the posterior region which had been underlain by mesoderm since the very beginning of gastrulation now lay at the anterior end of the embryo. If the regional determination of the nervous system depends upon the length of time for which neural ectoderm has had mesoderm beneath it, the operation should result in an abnormal nervous system, or one in reversed orientation.

Table 3

Total	30
Normal	10
Part normal remainder wounded	2
Reduced head	7
No head	1
Badly healed	10

The results were observed upon whole specimens. It was usually quite easy to recognize, by the general shape of the embryo, which had been the original posterior end, but in two experiments posterior halves of the presumptive neural plate were exchanged between pairs of embryos, one stained and one unstained, so that the differently coloured tissue marked the original anterior end in each embryo.

Table 4 summarizes the results of these operations. It can be seen that most of the embryos formed a nervous system with a reduced head: an example is shown in Text-fig. 3. Five had not formed a head at all. There were two cases (e.g. Text-fig. 4) where a tail as well as a reduced head had developed at the anterior end. Both of these had normal tails at the posterior end as well. The anterior tail must have been formed by self-differentiation of part of the presumptive posterior neural plate, while the rest of this region had developed 'neighbourwise' into anterior neural tissue.

Table 4

Total	19
Normal	2
Reduced head	8
No head	4
Second tail in anterior	2
Unusable	3

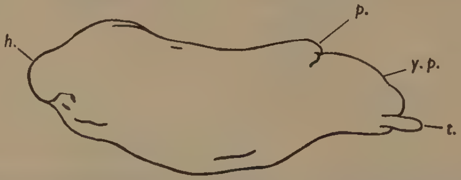
There were two embryos with practically normal nervous systems in the same orientation as the rest of their tissues.

In all these experiments the general polarity of the neural system was that of the main mass of the embryo, and in particular, that of the archenteron roof. It is clear, therefore, that the regional determination of the neural plate is not complete by the end of gastrulation, and cannot be solely dependent on the time of contact between ectoderm and mesoderm during the period when the latter is still moving.

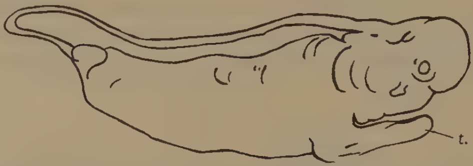
At the same time the defectiveness of the brains in several of the specimens, and the presence of a tail at the anterior end of one embryo, indicate that the regional determination has begun by the time the operation was performed, and that it is not usually possible for this to be completely reversed and a new 'regionality' fully imposed on the tissue in the period subsequent to that time.

§ 3. *Differentiation of presumptive ectoderm when placed directly into the archenteron roof*

Since the experiments described above gave no indication that a prolongation of the period of induction, for the length of time achieved, had any tendency to convert presumptive ectoderm into mesoderm, further experiments were done to investigate the general conditions which do bring about this result.



Text-fig. 3. Embryo with reduced head (*h.*) resulting from anterior-posterior reversal of dorsal ectoderm at late gastrula stage. *p.*, posterior edge of dorsal epidermis; *y.p.*, persistent yolk plug; *t.*, tail process.



Text-fig. 4. Formation of both head and secondary tail (*t.*) at the anterior end, after anterior-posterior reversal of dorsal ectoderm at late gastrula stage.

(*a*) *Presumptive ectoderm placed in the archenteron roof*

A piece of mesoderm one-fifth to one-quarter of the area of the archenteron roof was removed from a completed gastrula (H 12–12$\frac{1}{2}$), and replaced by an equal-sized piece of presumptive ectoderm from an early gastrula. The graft was stained in Nile Blue. Twenty-four transplants were carried out. Twenty-two of the hosts were sectioned after 5 days' culture, the graft having previously been seen in sixteen of them, occupying a dorso-lateral position beneath the epidermis. Two embryos were dissected to determine more exactly the differentiation of the graft; in one of these, it had formed the dorsal sectors of the somites in the posterior trunk and tail, and in the other (a late neurula) it occupied the dorsal and lateral walls of the anterior archenteron.

The position of the graft was identified in eighteen of the sectioned embryos, and in sixteen of these it had differentiated at least mainly into a neural tube. In a single instance, the implant formed epidermis in the roof of the gut. In all cases in which neural tissue had formed, there was some host mesoderm in the near neighbourhood of the implant, and it is reasonable to conclude that this induced the neural differentiation.

The Nile Blue was recognizable in only seven of the series of sections. In five of these the graft appeared to have formed neural tissue only, but in the two others, although the greater part of the implant had given rise to a neural mass, there were in its neighbourhood some blue-stained mesoderm cells which must have been derived from the peripheral parts of the implant. In some of the other cases in which the stain had disappeared during the preparation, the somites in the neighbourhood of the graft were enlarged, as though the implanted tissues had contributed to them. Further, in two embryos which were dissected during life, blue cells were definitely detectable in the mesoderm. It thus appears that small amounts of ectoderm, presumably from the peripheral parts of the implant, may become converted into mesoderm by this type of operation, although the bulk of it, and often the whole of it, is usually induced to neural tissue.

(*b*) *Presumptive ectoderm placed between archenteron roof and neural plate*

In a small series of experiments, only five in all, presumptive ectoderm from a young gastrula was placed between the archenteron roof and the overlying presumptive neural plate of a small yolk-plug host. In one of these, the implant formed neural tissue only but in the other four it became mainly converted into mesoderm which was partly incorporated into the host structures. The greater tendency for mesoderm to be formed in this type of experiment than in the last is probably to be attributed to the greater surface of contact between the implanted ectoderm and the host's archenteron roof.

(*c*) *Presumptive ectoderm made to invaginate into the archenteron roof*

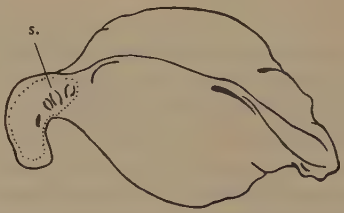
A piece of presumptive ectoderm from an early gastrula (H 10), stained in Nile Blue, was placed near the dorsal lip of a mid-gastrula (H 11–11½) so that it would invaginate into the posterior archenteron roof. Twenty-three operations were carried out and twenty-one embryos were sectioned at the gill-bud stage in the hope of tracing the fate of the graft. However, in most cases, it was impossible to do this; the graft had evidently been incorporated into the host tissue, but it was difficult to see where, because the blue colour did not show. More importance should therefore be attached to the observations on whole embryos, and to the obvious conclusion that the implants had as a rule undergone 'ortsgemäss' differentiation.

As far as possible, daily records were kept of the movements and differentiation of the transplanted tissue. In fourteen cases it was seen to occupy the posterior neural plate at the neurula stage, but in nine of these some graft tissue had also invaginated. In four cases the invaginated parts had definitely formed somites (Text-fig. 5); in five others some of the tissue was visible in the somite region, but its differentiation was not clear.

In ten embryos the grafts invaginated completely. One of these formed a secondary axis (possibly with the inclusion of some host tissue), and another was visible in the somite region of the host, but the differentiation of the remaining seven could not be seen.

Of the two grafts which could be certainly identified in sections, one had formed a mass of dead cells in the gut lumen, and the other, a neuroid thickening in the host ectoderm. There was no further evidence of the graft having formed ectodermal tissue.

There were, however, several cases in which the graft had probably formed mesoderm, since supernumerary mesodermal structures could be observed. For instance, two embryos showed a secondary notochord, and in one of these it was some distance from the primary notochord so that it did not appear to be derived from this. There were also eight embryos in which the somites appeared much larger on one side of the body and one in which there was an extra somite block on one side. In two embryos, where the graft had been seen to invaginate at the lateral instead of the dorsal lip, enlarged kidney tubules could be observed on one side of the trunk.



Text-fig. 5. Embryo in which grafted ectoderm had formed both neural tube and somites (*s.*), seen prior to dissection. Area occupied by the graft indicated by dotted outline.

One may conclude from these results that the implanted ectoderm never underwent self-differentiation. Parts of it which remained on the surface were seen to differentiate into neural plate according to their position on the host, and parts which had invaginated were never seen either to form abnormal structures or to migrate outwards to the surface. In three cases they may have formed the supernumerary axial mesoderm which was observed, and in other cases it must be presumed that they became incorporated into the mesoderm of the host.

DISCUSSION

In the experiments described above, the results of prolonging the contact between the presumptive neural plate and the underlying archenteron roof have been very slight. In no cases did this succeed in converting the tissue into mesoderm, and there were only slight indications that it had any effect on the 'regionality' of induced neural material. There could be two reasons for this; either that the duration and intensity of the inducing stimulus does not have an appreciable effect on the nature of the result, which is determined in some other way, or that the experiments did not in fact materially increase the period of effective action of the archenteron roof. There are several considerations which suggest that the second alternative may be the correct one. In the experiments described in § 2*a*, pieces of presumptive ectoderm which had not undergone the early period of gastrulation were able to develop into part of a normal neural axis under the influence of the late gastrular organizer alone. This suggests that the intensity of the inducing action is low in the early stages of gastrulation. It is true that the results under § 2*b* show that the action is not completely absent even in the early stages, and that a certain degree of regional determination is conferred on the presumptive ectoderm and cannot always be completely reversed during the later gastrula stages; but the fact that at least partial reversal occurs in these experiments only reinforces the conclusion that the inducing action is stronger in the later than in the earlier period. Thus when, in the experiments of § 1*a* and *b*, late gastrula neural plate is removed and grafted into an early gastrula, it is at first placed into an environment of weaker inducing action than that from which it was taken. The total 'dose' of induction to which it has been submitted will only begin to rise above the normal dose towards the end of the gastrulation of its new host. The question then arises whether this may not be too late to be effective. The grafts tend to begin differentiating at a time dependent on their own age, not on the age of their young hosts; and when they start differentiating, if not at some rather earlier time, their basic regional determination must be complete. Thus it seems quite probable that the effective dose of induction was not actually raised during the period within which the grafts were capable of being influenced by it.

This conclusion does not apply so forcibly to the experiments described under § 1*c*. In these, a young gastrula ectoderm was given an initial period of the inducing influence of the mid to late gastrula mesoderm, which is presumably fairly powerful in action; it was then transferred to a young gastrula and went through the normal gastrulation process. If it retained effective competence throughout the whole of this, its total dose of induction would have been raised by the amount represented by its period in its first host. It is doubtful, however, whether this is entirely the case, since the grafts were observed to begin rolling into a neural groove at a time when the host tissues surrounding them were still in the open neural plate stage. This suggests that the ageing of the graft effectively cut short the final period of inductive action, and thus to some extent reduced the total dose below the possible maximum described above. Nevertheless, there was as we have seen some, if rather slight, indication that the 'regionality' of the graft was shifted towards a more posterior character.

The experiments described in this paper therefore do not suffice to disprove the suggestion made by Waddington & Yao (1950) that the duration and intensity of induction play an important role in regional determination, although they provide little support for it. The studies of Gallera (1947) and Damas (1947), in which the dose of induction was reduced, have also thrown little light on the matter. They were able to show that tissue, which if left in place would have developed into a derivative of the medial region of the neural plate could, if precociously removed from the inductive influence, differentiate into a derivative of the lateral parts of the plate (i.e. into ectomesoderm), but they describe no definite evidence of a change in the antero-posterior level of their grafts, and it is doubtful if such a change could have been recognized in the very small grafts they employed. It remains necessary to find a more penetrating experimental method with which to attack the problem.

The same remark applies to the induction of mesoderm, in regard to which the experiments have only succeeded in clearing up a few points. It has been known since the early studies of Bytinski-Salz (1929) that presumptive ectoderm of the early gastrula, when placed within the blastula, may become converted into mesoderm, although it is frequently induced to develop into neural tissue. Since Bytinski-Salz placed his implants freely within the blastocoel cavity, it was not clear what situation they occupied at the time when their determination occurred. In § 3*a* and *b*, we have shown that if gastrula ectoderm is substituted for a piece of the archenteron roof it always becomes neural tissue, except that a few cells at the edges of the graft may be converted to mesoderm, while if the ectoderm is placed between the archenteron roof and the overlying neural plate, it much more frequently develops into mesoderm. The former result shows that there is no general condition of the interior of the gastrula which can effectively cause all competent tissue exposed to it to become mesoderm. This inductive effect, in fact, appears to be exerted by the invaginating mesoderm itself. It is probably significant that in the experiments of § 3*a*, in which the ectoderm is only in contact with mesoderm along its edges, only neural tissue is induced, whereas in those of § 3*b*, in which the contact is over the whole area of the transplant, the graft may become mesoderm. This suggests that mesoderm can only be formed if a rather high dose of inductive action is applied to the ectoderm. In the experiments of § 3*b*, however, the doses can hardly be higher than those which, in other circumstances, give neural inductions. It remains obscure, however, why ectoderm placed over the archenteron roof becomes neural plate when it is on the surface of the gastrula, but tends to become mesoderm when it is below the surface and covered by a second layer of ectoderm as it was in these experiments. It seems after all that there must be some crucial importance in the ectoderm being either inside the egg or on its surface (cf. Lehmann, 1945).

This conclusion is reinforced by the results of the experiments in § 3*c*, in which, repeating Mangold (1923), Spemann & Geinitz (1927), Töndury (1936) and Raven (1938), we have shown that ectoderm placed within the presumptive mesoderm of the early gastrula so as to invaginate through the blastopore, nearly always becomes converted into mesoderm. During the invagination it has, of course, been underlain by the anterior mesoderm which moves first into the interior of the embryo, and has therefore had close contact with inducing material over its whole surface. It has thus undergone a treatment similar to that of the ectoderm in § 3*b*. But the same is true of ectoderm which remains on the surface and becomes induced to neural tissue. Moreover, the intensity and duration of the inducing action to which the grafts in § 3*b* were submitted would be less than that applied to the § 3*c* grafts which invaginate along with the mesoderm, and similar to that of the neural plate itself. This strongly suggests that in the decision whether an ectodermal graft

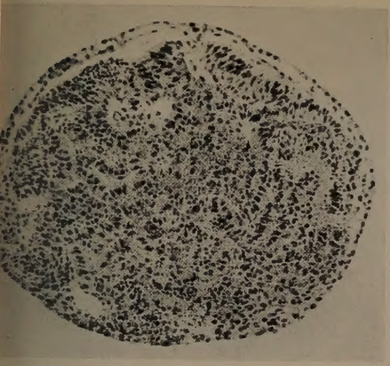
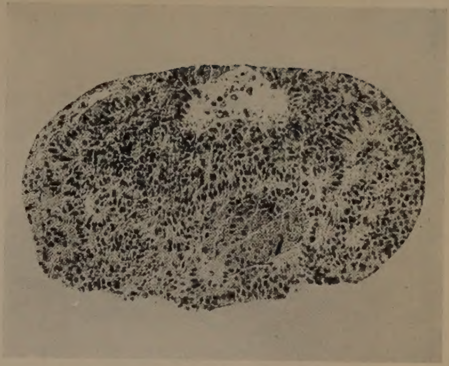


Fig. 1.



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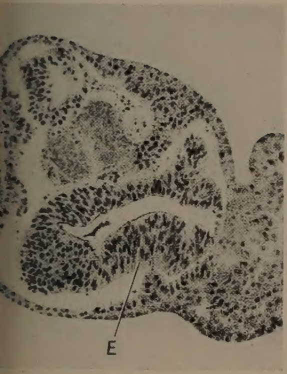


Fig. 3.

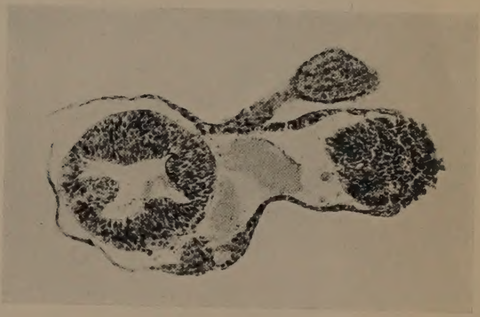


Fig. 4.

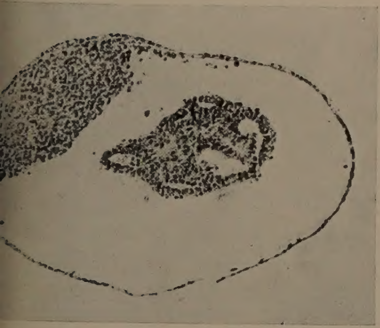


Fig. 5.

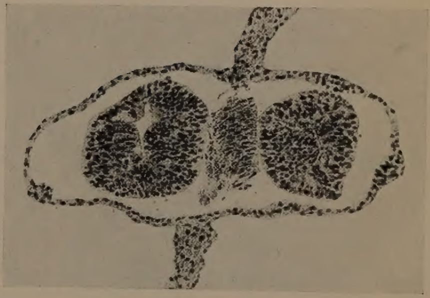


Fig. 6.

VADDINGTON AND DEUCHAR—EFFECT OF TYPE OF CONTACT WITH ORGANIZER ON INDUCTION

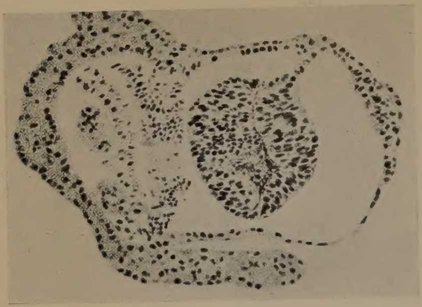


Fig. 1.

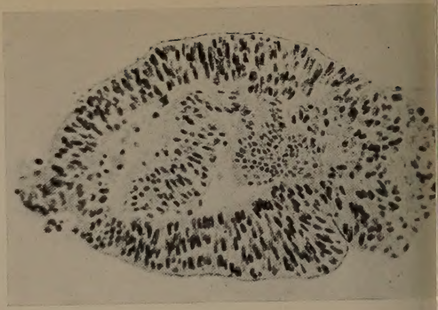


Fig. 2.

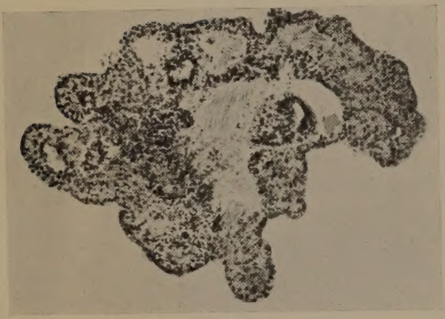
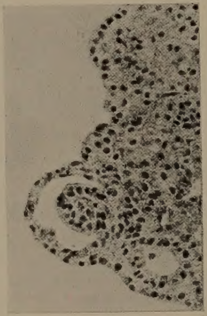


Fig. 3.



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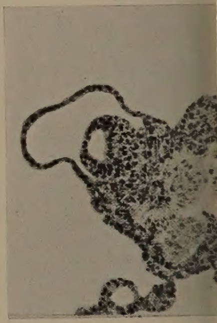

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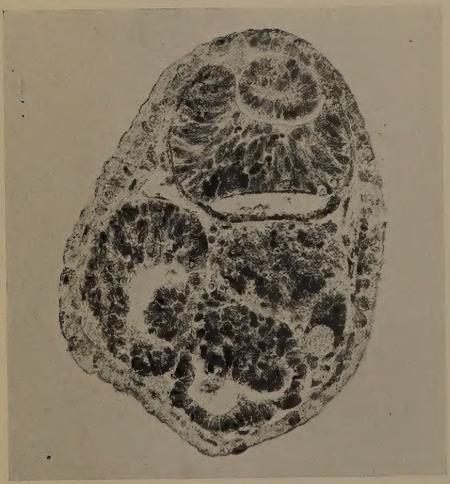

Fig. 6.

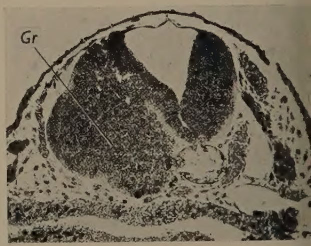

Fig. 7.

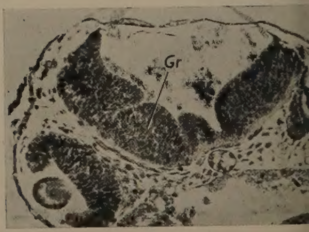

Fig. 8.

WADDINGTON AND DEUCHAR—EFFECT OF TYPE OF CONTACT WITH ORGANIZER ON INDUCTION will develop as neural plate or as mesoderm a more important part is played by the position of this graft on the surface or beneath the surface than by the total dose of induction it has undergone (provided that the dose passes some threshold which is not achieved in the grafts made as in § 3*a*). It still remains uncertain whether it is possible, by increasing the effective dose of inducing action, to cause tissue, which is still on the surface of the gastrula at the time of the open neural plate stage, later to differentiate into axial mesoderm.

SUMMARY

1. An attempt was made to increase the effective 'dose' of inducing action by transplanting pieces of presumptive neural plate from late gastrula stages into the presumptive neural plate of younger hosts, so that they were exposed to the action of the archenteron roof a second time. No difference was seen in their mode of development, either when they were left in the second embryo or if they were explanted from it (together with the underlying mesoderm) into salt solution.

2. If a young gastrula ectoderm is submitted to the action of the archenteron roof for the period from the mid- to late-gastrula stages, and then transplanted to a new young gastrula, it develops morphologically in conformity with its host, but there is some slight indication that the histology of the tissue is characteristic of a rather more posterior level of the neural system than that in which it lies.

3. No method of increasing the dose of inductive action succeeded in producing the slightest tendency for tissue lying in the presumptive neural plate of the late gastrula to develop into mesoderm.

4. If the whole presumptive neural plate is removed from a late gastrula, and ectoderm from a young gastrula substituted for it, normal regional differentiation may occur, in spite of the fact that all parts of the grafted ectoderm will have been underlain by inducing material for the same length of time.

5. If the presumptive neural plate of a late gastrula is removed and then replaced with its anterior-posterior axis reversed, most of the embryos have a reduced head or none at all, and a tail may form over the anterior mesoderm. Thus, although it can be recognized that the main polarity of the embryo has been determined by the mesoderm, there is considerable evidence that regional determination has progressed some way during the period of invagination. The results summarized in the previous paragraph show, however, that the effects of this early period of labile regional determination are not indispensable.

6. If gastrula ectoderm is grafted directly into the archenteron roof, it nearly always develops into neural tissue, whereas if it is placed between the archenteron roof and the overlying neural plate, it often differentiates into mesoderm. This is held to demonstrate that there is no condition of the interior of the gastrula which directly converts ectoderm into mesoderm; that the induction of mesoderm requires a direct contact of the whole surface of the ectoderm with inducing mesoderm; and that, provided a certain minimum intensity of inducing action has been achieved, the decision whether the ectoderm will become mesoderm or neural plate depends on whether it lies on the surface of the embryo or beneath it.

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EXPLANATION OF PLATES

All figures are sections of sheets of gastrula ectoderm into which heat-coagulated fragments from the embryonic axis of neurula and tail-bud embryos have been grafted.

PLATE 20

Fig. 1. F25*b*. Graft from anterior end of open neural plate stage, fixed after 4 days. A mass of contorted neural tubules. (× 55.)

Fig. 2. F24*b*. Similar, graft from posterior end. (× 55.)

Fig. 3. F75*b*3. Graft consisted of eyes and brain from late tail-bud stage. Fixed after 6 days. Type 1. (× 70.) *E*, induced eye.

Fig. 4. J13*a*. Graft, brain from mid tail-bud stage. Fixed after 7 days. Type 1. (× 70.)

Fig. 5. H20*b*4. Graft, brain from Axolotl mid tail-bud. Fixed after 9 days. Type 2. (× 55.)

Fig. 6. M26*b*1. Graft, brain of late tail bud. Fixed after 6 days. Type 3. (× 60.)

PLATE 21

Fig. 1. M27*b*4. Graft, posterior end of axis of mid tail bud. Fixed after 6 days. Type 3. (× 80.)

Fig. 2. M26*b*5. Graft, brain from late tail bud. Fixed after 6 days. Type 5. (× 100.)

Fig. 3. J12. Graft, posterior end of axis of mid tail bud. Fixed after 9 days. Type 6. (× 70.)

Fig. 4. M37*b*. Graft, brain of late tail bud. Fixed after 7 days. Type 7. (× 80.)

Fig. 5. M76*b*1. Graft, posterior end of open neural plate stage. Fixed after 9 days. Type 7. (× 55.)

Fig. 6. Formation of brain and eye by presumptive anterior neural plate explanted after having been subjected to prolonged organizer action. (× 135.)

Fig. 7. A graft of 'doubly underlain' ectoderm (*Gr*) lies in the wall of the hind-brain, but appears histologically more like the neural tube of the trunk. (× 55.)

Fig. 8. Another case similar to fig. 2. (× 55.)